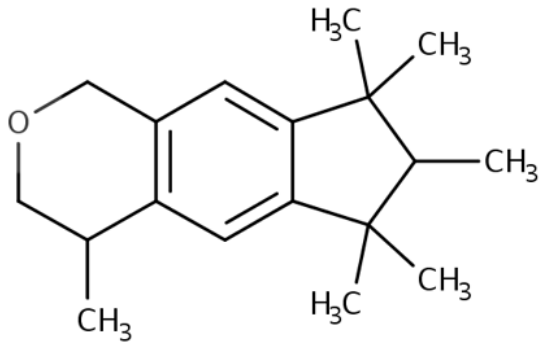
United States
Environmental Protection Agency

March 2026
Office of Chemical Safety and
Pollution Prevention

Draft Data Evaluation Records for Human Health Hazard for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta [g]-2-benzopyran (HHCB)

Support Document for the Draft Risk Evaluation

CASRN 1222-05-5



March 2026

1 Introduction

This supplemental file contains the data evaluation records (DERs) for human health hazard studies that were used either quantitatively or qualitatively as weight of evidence in the *Draft Human Health and Environmental Hazard Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026). Table 1 lists these studies in the order that they are evaluated:

Table 1. Studies Reviewed in this Supplement

Number	Title	Reference
1	Extended one-generation reproductive toxicity study (including cohorts 1 and F2 – generation of HHCB by the oral route (dietary admixture) in the rat (OECD 443) (sanitized)	(IFF, 2021)
2	AHTN and HHCB show weak estrogenic – but no uterotrophic activity	(Seinen et al., 1999)
3	HHCB no. 24. Repeated insult patch test (sanitized)	(IFF, 1973a)
4	Repeated patch test. Galaxolide (sanitized)	(IFF, 1964)
5	Repeated insult patch test galaxolide 50 (sanitized)	(IFF, 1973b)
6	The systemic exposure to the polycyclic musks, AHTN and HHCB, under conditions of use as fragrance ingredients: Evidence of lack of complete absorption from a skin reservoir	(Ford et al., 1999)
7	In-vitro human skin penetration of radiolabeled fragrance material HHCB (redacted)	(An-eX, 2001)
8	HHCB/Galaxolide undiluted: In vitro Episkin (tm) skin irritation test (sanitized)	(IFF, 2020)

References

An-eX. (2001). In-vitro human skin penetration of radiolabelled fragrance material HHCB (redacted). (RIFM/4/00). Woodcliff Lake, NJ: Research Institute for Fragrance Materials Inc.

Ford, RA; Hawkins, DR; Schwarzenbach, R; Api, AM. (1999). The systemic exposure to the polycyclic musks, AHTN and HHCB, under conditions of use as fragrance ingredients: Evidence of lack of complete absorption from a skin reservoir. Toxicol Lett 111: 133-142. https://dx.doi.org/10.1016/S0378-4274(99)00174-5

IFF. (1964). Repeated patch test. Galaxolide. (sanitized). New York, NY.

IFF. (1973a). HHCB no. 24. Repeated insult patch test (sanitized). New York, NY: International Flavors & Fragrances ::IFF.

IFF. (1973b). Repeated insult patch test galaxolide 50. (sanitized). New York, NY: International Flavors & Fragrances :: IFF.

IFF. (2020). HHCB/galaxolide undiluted: In vitro Episkin (tm) skin irritation test. (sanitized). New York, NY: International Flavors & Fragrances :: IFF.

IFF. (2021). Extended one generation reproductive toxicity study (including cohorts 1 and F2 - generation of HHCB by the oral route (dietary admixture) in the rat (OECD 443) (sanitized). New York, NY: International Flavors & Fragrances :: IFF.

Seinen, W; Lemmen, JG; Pieters, RH; Verbruggen, EM; van der Burg, B. (1999). AHTN and HHCB show weak estrogenic--but no uterotrophic activity. Toxicol Lett 111: 161-168. https://dx.doi.org/10.1016/s0378-4274(99)00177-0

U.S. EPA. (2026). Draft Human Health and Environmental Hazard Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB). Washington, DC: Office of Pollution Prevention and Toxics.

DATA EVALUATION RECORD

HHCB (GALAXOLIDE)

Study Type: OCSPP 870.3800; Extended One-Generation Reproductive Toxicity Study in Rats

EPA Contract No. 68HERC22D0017
Task Assignment No. 5542-2.1-010 (MRID # not provided)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
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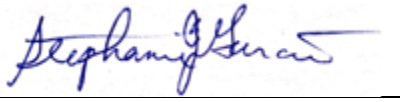
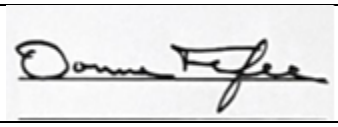
Austin, TX 78746

Primary Reviewer:	Signature:	
Stephanie J. Garcia, Ph.D.	Date:	05/02/2025
Secondary Reviewer:	Signature:	
Donna Fefee, D.V.M.	Date:	05/16/2025
Quality Assurance:	Signature:	
Scott D. Studenberg, Ph.D., DABT	Date:	05/29/2025
Project Manager:	Signature:	
Scott D. Studenberg, Ph.D., DABT	Date:	05/29/2025

Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by PB&A/CSS Joint Venture personnel. Contractor's role did not include establishing Agency policy.

EPA Reviewer: Greg Akerman **Signature:** _____
Immediate Office, HED (7509T) **Date:** _____
EPA Secondary Reviewer: _____ **Signature:** _____
Immediate Office, HED (7509T) **Date:** _____

Template version 12/23

TXR: Not available

DATA EVALUATION RECORD

STUDY TYPE: Extended One-Generation Reproductive Toxicity Study - Rats; OCSPP 870.3800; OECD 443.

PC CODE: Not available **DP BARCODE:** Not available

TEST MATERIAL (PURITY): HHCB (Galaxolide) (0.12% Galaxol [0.01% Galaxolide D and 0.01% Galaxolide L])

SYNONYMS: 1,3,4,6,7,8-hexahydro- 4,6,6,7,8,8- hexamethylindeno [5,6-*c*]pyran; 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8,-hexamethyl-cyclopenta[*g*]benzopyran; galaxolide

CITATION: Mounier, R. (2021) Extended one-generation reproductive toxicity study (including cohorts 1 and F2-generation) of HHCB by the oral route (dietary admixture) in the rat (OECD 443). Test Facility, Study Number, and Date redacted. MRID not provided. Unpublished.

SPONSOR: International Flavors & Fragrances I.F.F.B.V., Hilversum, Netherlands

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: In an extended one-generation reproductive toxicity study (MRID # not provided), groups of 25 Wistar (Crl:WI[Han]) rats/sex were administered HHCB (Galaxolide; 0.12% Galaxol [0.01% Galaxolide D and 0.01% Galaxolide L]; Batch #: 0010340146) in the diet at concentrations of 0, 470, 825, or 1650 ppm during premating, mating, and gestation (equivalent to 25.8/29.0, 45.9/50.6, and 94.1/99.8 mg/kg/day in P males/females during premating) and 0, 205-340, 365-600, and 730-1200 ppm (equivalent to 34.4, 57.5, and 116.3 mg/kg/day in the P dams) during lactation (doses were altered weekly) up to lactation day (LD)/postnatal day (PND) 21. The P generation animals were fed the test diets for a minimum of ten weeks prior to mating to produce

the F1 litters; the P males were terminated after a minimum of twelve weeks of dosing and the P females were terminated after weaning on LD 22-24. At weaning, the F1 generation pups were divided into Cohorts 1A, 1B, and 1C (containing 20 pups/sex/treatment level/cohort) and fed the same dietary concentrations as their parents. Cohort 1A animals were assessed for clinical pathology, time between vaginal patency and onset of estrus, estrous cycle data, differential ovarian follicle counts, sperm parameters, and splenic lymphocyte subpopulation analysis, and were terminated on PND 8593. Cohort 1B offspring were mated to produce F2 litters, assessed for reproductive parameters, and terminated after mating (males) or on LD 21-23 (females). F1 offspring from all cohorts were assessed for vaginal opening and preputial separation, and Cohort 1C animals were terminated after positive identification of these landmarks. Some unselected F1 offspring were used for assessment of thyroid-related hormones and organ weights (on PND 21), with termination on PND 21-23. Selected culled PND 4 pups were also used for hormone assessment. In addition to standard assessment of litter parameters for F1 and F2 offspring, F2 pups were also examined for anogenital distance (AGD) on PND 1 and were terminated on PND 21-23.

There were no treatment-related effects on mortality; clinical signs of toxicity; hematology, clinical chemistry, and urinalysis parameters; splenic lymphocyte subpopulations; and macroscopic pathology. Treatment-related decreases in body weight, body weight gain, and food consumption were observed in the ≥825 ppm adult F1 males throughout the study and in the 1650 ppm adult F1 females during gestation. Observed thyroid-related effects included increased absolute and/or relative thyroid weights in the 1650 ppm P and F1 males and females, the 825 ppm P females and F1 males, and the 430 ppm P females and F1 Cohort 1A males; increased minimal to mild bilateral diffuse follicular cell hypertrophy in the ≥825 ppm P and the 1650 ppm F1 animals of both sexes; and decreased serum T4 concentrations in the ≥825 ppm P and F1 males and increased ($p<0.05$) TSH in the ≥825 ppm F1 females. Based on the absence of treatment-related lesions in the thyroids and liver weight increases in the 1650 ppm animals (attributed to an adaptive response), the thyroid-related effects are considered as liver-mediated (*e.g.*, treatment-related induction of hepatic enzymes). Therefore, these changes were considered non-adverse.

The parental LOAEL for dietary HHCB in Wistar rats was 825 ppm (equivalent to 45.9/50.6 mg/kg/day in the P males/females during premating) based on decreases in body weight. body weight gain, and food consumption in the F1 males. The NOAEL was 470 ppm (equivalent to 25.8/29.0 mg/kg/day in the P males/females during premating).

There were no adverse, treatment-related effects on viability; clinical signs of toxicity; TSH concentrations; and macroscopic pathology. Treatment-related decreases in pup body weight were evident in offspring at ≥365-600 ppm of both generations. These decreases were reflected in the concomitant body weight gain values and considered adverse. Preputial separation was delayed at all treatment-levels in a manner unrelated to treatment level but the delays were approximately 1.5-2.5 days greater than the concurrent control and approximately 4-11 days greater than the HCD range. The delay in the 730-1200 ppm males occurred concurrently with decreased body weight. Serum TSH concentrations were increased ($p<0.05$) in the 730-1200 ppm F1 males and the 365-600 ppm F1 females on PND 21 (observed effects in the F1 PND 21 females were consistent with the observed effects in the F1 Cohort 1A adult females. As with adults, these differences are likely related to treatment but not adverse.

The offspring LOAEL for dietary HHCB in Wistar rats was 825 ppm (equivalent to 45.6/57.5 mg/kg/day in the P females during gestation/lactation) based on decreased body weights and body weight gains in F1 and F2 offspring. The NOAEL was 470 ppm (equivalent to 26.8/34.4 mg/kg/day in the P females during gestation/lactation).

There were no adverse treatment-related effects on estrous cycle parameters in the P and F1 Cohort 1A/1B females; sperm parameters in the P or Cohort 1A males; mating/fertility indices, pre-coital interval, gestation index in the P or F1 Cohort 1B animals; or differential ovarian follicle count evaluations in the F1 Cohort 1A females. In the F2 offspring, the AG index was decreased by 4% in the 205-340 ppm males and decreased by 16-21% and 22-26% in the ≥365-600 ppm males and females, respectively. Data pertaining to AG indices represent a reproductive effect, and as biomarkers of endocrine-mediated effects, were considered adverse.

The reproductive LOAEL for dietary HHCB in Wistar rats was 825 ppm (equivalent to 45.6/57.5 mg/kg/day in the P females during gestation/lactation) based on decreased anogenital indices in F2 males and females. The NOAEL was 470 ppm (equivalent to 26.8/34.4 mg/kg/day in the P females during gestation/lactation).

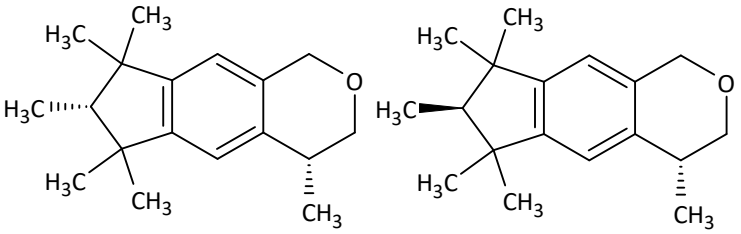
This study is classified **Acceptable/Guideline** and satisfies the requirements for an extended one-generation reproductive toxicity study (OCSPP 870.3800; OECD 443) in the rat. Note to EPA Reviewer: The Reviewers note that the bases for omitting the developmental neurotoxicity and immunotoxicity cohorts were not provided, and some information required for study reports was redacted.

COMPLIANCE: Signed and dated GLP Compliance and Quality Assurance statements were provided; however, the names and dates were redacted. All pages of the report included the following information in a footer: “The posting of this document to a public docket maintained by a governmental agency is not a waiver of ownership rights of International Flavors and Fragrances (IFF). Any use of the document by any other person without express written permission would constitute a violation of IFF's rights and subject that person to civil liability. Redactions are claimed as confidential business information (CBI) in accordance with 40 C.F.R. Section 702.37(d).”

I. MATERIALS AND METHODS:

A. MATERIALS:

1. Test material: HHCB (Galaxolide)

Description:	Colorless viscous liquid
Batch #:	0010340146
Purity:	0.12% Galaxol (0.01% Galaxolide D and 0.01% Galaxolide L)
Expiration/Storage:	May 21, 2021/Controlled room temperature
CAS Nos. of TGAI:	1222-05-5; 252933-49-6 (Constituent 1); 252933-48-5 (Constituent 2)
Structures:	

Constituent 1 Constituent 2

2. Vehicle: The test article was administered via the basal diet (Diet Reference No. A04C-P2.5, Scientific Animal Food & Engineering (SAFE); Batch/Lot Nos. 19065, 19268, 19330, 20041, and 20113).

3. Test animals

Species:	Rat	
Strain:	Wistar (Crl:WI[Han])	
Age at study initiation:	P males and females: approximately 6 weeks	
Body weight at study initiation:	P males: 145.3-204.3 g; P females: 104.4-154.9 g	
Source:	Redacted	
Housing:	5 rats/sex/cage in plastic cages, except as noted, with cellulose bedding or dust-free sawdust. Enrichment materials (wooden gnawing blocks and shredded paper) were provided. Mating: 1 male and 1 female co-housed Gestation: females housed individually Lactation: dam housed with litter until weaning (PND 21).	
Diet:	Irraduated rat powdered complete diet (Diet Reference No. A04C-10, SAFE), *ad libitum*.	
Water:	Softened and filtered (0.2 µm) drinking water, *ad libitum*.	
Environmental conditions:	**Temperature:**	19-25°C
	Humidity:	>35%
	Air changes:	≥10/hour
	Photoperiod:	12 hours light:12 hours dark (except during designated procedures)
Acclimation period:	7 days	

B. PROCEDURES AND STUDY DESIGN:

1. In-life dates: Redacted.

2. Mating procedure: After a minimum of 10 weeks of treatment, mating of parental (P) and F1 Cohort 1B animals was accomplished by co-housing one female with one male from the same dose

group for up to 14 consecutive days. Mating was detected by evidence of sperm in the daily vaginal smear or by the appearance of an intravaginal copulatory plug, and the day on which mating was detected was designated as gestation day (GD) 0. After confirmation of mating, females were housed individually. Females without evidence of mating were separated from their paired male when their appearance suggested pregnancy from an undetected mating.

3. **Study schedule:** The test material was administered continuously at nominal dietary concentrations as indicated in Table 1 to groups of 25 healthy P generation rats/sex for at least ten weeks prior to mating, during mating (up to two weeks), during gestation and lactation (as applicable, for females) and up to the day prior to necropsy. Males were euthanized after a minimum of 12 weeks of dosing. Females that delivered litters were euthanized between Lactation Day (LD) 22-24. Females that failed to litter were treated for at least 23 days after the last day of the mating period; those with evidence of mating were terminated on GD 26, and those without evidence of mating were terminated approximately 26 days after the last day of the mating period. Females with total litter loss were euthanized within 24 hours. During lactation, the dams were provided diets with reduced test substance concentrations to account for their greater food consumption during this interval; the adjustments were based on historical food consumption and body weight data.

   On postnatal day (PND) 21, selected F1 pups were allocated to one of three cohorts (1A, 1B, or 1C) to achieve 20 animals/sex/group/cohort; these animals were fed the same diets as their parents and treated continuously up to the day prior to or the day of necropsy. Surplus F1 animals were not treated and were terminated on PND 21; a subgroup was used for hormone analysis. Cohort 1C animals were euthanized after sexual maturation. Cohort 1A animals were euthanized between PNDs 85 and 93 and appropriate samples were collected for clinical pathology, sperm and estrous parameter, and microscopic pathology evaluations.

   Cohort 1B animals were treated for a minimum of 10 weeks and then mated to produce F2 offspring; males were euthanized after at least 20 weeks of exposure and females after at least 19 weeks of exposure. The F2 offspring were euthanized on PND 21-23.

4. **Animal assignment:** After arrival and inspection for health, the parental (P) animals were randomly assigned to the groups indicated in Table 1; randomization procedures were not detailed. F1 animals were allocated into cohorts at the time of weaning (PND 21); the selection and allocation procedures were not described.

TABLE 1. Animal assignment. [a]

Test group	Nominal dietary concentration (ppm) [b]	Animals/group			
		P	F1		
			Cohort 1A	Cohort 1B	Cohort 1C
Control	0	25/sex	20/sex	20/sex	20/sex
Low	470	25/sex	20/sex	20/sex	20/sex
Mid	825	25/sex	20/sex	20/sex	20/sex
High	1650	25/sex	20/sex	20/sex	20/sex

a Data were obtained from page 17 and Text Table 6 on page 31 of the study report.

b Exposure to the test substance was continuous throughout the study. Dietary concentrations fed to lactating females were decreased in the low-, mid-, and high-dose groups as follows: LD 1-7 (340, 600, and 1200 ppm); LD 8-14 (255, 450, and 900 ppm); LD 15-21 (205, 365, and 730 ppm).

5. Dose-selection rationale: Dose levels were based on a 90-day repeated-dose study, a prenatal developmental toxicity study, and a dose range-finding study conducted for the current study. iIncreased liver weights were apparently observed at dose levels of 62.5 mg/kg/day (based on the dose range-finding study) and 150 mg/kg/day (based on the 90-day repeated-dose study). Although no treatment-related effects on fetal weight were observed in the prenatal developmental toxicity study up to 150 mg/kg/day,. , pup weights were decreased less than the historical control data range on PNDs 4 and 14, but not PND 21, in the dose range-finding study at 225 mg/kg/day. Pup weights were within historical control data levels, and no effects on fertility were noted (assumed at all dose levels by the Reviewers). Based on the observed systemic effects, target dose levels of 30, 60, and 120 mg/kg/day were selected.

Based on the results of previous stability analyses, dietary concentration decreases of approximately 25% were expected over the course of four days. To compensate for these anticipated losses, target doses for the current study were increased accordingly by 25%: 42.5, 75, and 150 mg/kg/day. Note to EPA Reviewer: The difference between 30 and 42.5 mg/kg/day was approximately 42% instead of 25% (37.5 mg/kg/day). Based on the expected body weight gains and food consumption of the animals and historical control data for females during lactation, the selected target dietary concentrations were 470, 825, and 1650 ppm, with adjustments as follows: 340, 600, and 1200 ppm for dams during LD 1-7; 255, 450, and 900 ppm for dams during LD 8-14; and 205, 365, and 780 ppm for dams during LD 15 to 21-23.

6. Test diet preparation and analysis: The test item was prepared as a dietary admixture. The test item was stored at 60-70°C for a minimum of 24 hours, mixed well, and heated, if necessary, to liquify. For each concentration, an appropriate amount of test item was weighed and combined with a small amount of diet in a blender to produce a pre-mixture. The pre-mixture was then diluted with an appropriate quantity of basal diet to achieve the desired concentration and mixed further. Test diets were formulated weekly and divided into two portions; one was stored refrigerated (2-8°C) and the other was stored at room temperature (15-25°C).

Quadruplicate samples of the test diets were collected and stored at −20°C prior to analysis for homogeneity, concentration, and/or stability as follows. Samples for homogeneity were taken from the right, middle, and left positions on Day 1 (470 and 1650 ppm) and from the top, middle, and bottom positions during Week 33 (205 and 1650 ppm); averaged results from these samples

were used for concentration verification. Additional samples for concentration analysis were taken from the middle of the preparations and analyzed as follows: Day 1 (0 and 825 ppm), Week 11 and Week 22 (all diet concentrations), Week 16 (205 and 1650 ppm), and Week 33 (0 ppm). Stability was determined during Week 16 after storage at 2-8°C for four days or storage at 15-25°C for four or eight days.

Results:

Homogeneity analysis (% RSD): 1.6-3.2%.

Stability (% initial analysis): 91.3-92% or 84.688% of baseline after storage for four or eight days at room temperature, respectively, and 93.7-93.8% after refrigerated storage for four days (assuming Week 16 concentrations as the initial concentrations). Note to EPA Reviewer: Although the administered doses were increased by 25% to account for test material instability, this increase was based on decreases relative to the nominal concentrations instead of the observed decreases relative to the initial values (maximum decreases of approximately 15%).

Concentration (mean % nominal): 81.6-100.3%; Test material was detected in duplicate control samples on 1 of 4 occasions (Week 33) at 10.86 ppm.

The analytical data indicated that the mixing procedure was adequate, and that the variance between nominal and actual dosage to the study rats was acceptable.

7. **Dosage administration:** The test material was administered in the diet continuously for ten weeks prior to mating and throughout mating, gestation, and lactation until scheduled termination. The control groups were administered basal diet.

C. OBSERVATIONS:

1. **Parental/adult rats:** The following observations were made for all P and F1 generation (Cohorts 1A, 1B, or 1C) animals (unless noted otherwise). Animals were observed at least twice daily (at least once daily on days of receipt and necropsy) for morbidity and mortality. Cage-side clinical observations were conducted at least once daily, and a full clinical evaluation was performed at least on each day of weight collection. Time of onset, grade, and duration of any observed signs were recorded, if applicable.

   Body weights were determined on the first day of the premating period (P animals) or on the first day post-weaning (Day 1 [PND 22]; Cohorts 1A, 1B, 1C) and then at least weekly thereafter. In addition, body weights of mated P and F1 (Cohort 1B) females were recorded on GDs 0, 4, 7, 11, 14, 17, and 20 and on LDs 1, 4, 7, 10, 14, 17, and 21. Food consumption was determined twice weekly for P and F1 animals. Additionally, food consumption of mated P and F1 Cohort 1B females was recorded daily during gestation and lactation and presented for the intervals corresponding to days body weights were recorded. Food consumption was not determined during the mating period or for females without evidence of mating.

To evaluate estrous cycle length and regularity, daily vaginal lavages were conducted in the P females beginning 14 days prior to mating and continuing until evidence of copulation was observed (or until the end of the mating period) and in the F1 Cohort 1A females for at least two weeks prior to necropsy. The presented data for each female included the animal's mean estrous cycle length, irregularity index, and the percentage of days in estrus or a notation that the animal was acyclic or considered to have "acyclic period." In addition, vaginal lavages were performed daily for the F1 Cohort 1A females beginning on the day of onset of vaginal patency and continuing until the first estrus was observed. P generation females, and females in F1 Cohorts 1A and 1B, also had smears taken on the day of scheduled necropsy (or from moribund animals in the case of the P generation females).

The pairing partners, the number of mating days until mating was confirmed, and gestational status were recorded for P and F1 Cohort 1B males and females. Mating, fertility, gestation, and live birth indices and the percentage of post-implantation loss were calculated. The P and F1 Cohort 1B females were observed daily during the end of gestation for signs of parturition; the day of occurrence was designated as LD 0 (dams) or PND 0 (offspring).

2. **Litter observations:** Litter parameters (X) were recorded according to Table 2. Litters were examined on the day of birth (PND 0) to determine the total number of pups born and the numbers of live/stillborn pups; the sex of each pup was recorded on PND 1. F1 and F2 pups were examined for mortality or morbidity at least twice daily during the cage-side observations of each dam and for clinical signs at least once daily. Live pups were individually weighed on PNDs 1, 4, 7, 14, and 21. The sex of each pup was determined externally on PNDs 1 and 4. Anogenital distance (AGD) was measured for live F1 and F2 pups on PND 1 and normalized to the cube root of body weight (AG index [mm/kg]). On PND 4, litters with more than eight pups were standardized to eight (four/sex, if possible); selective elimination was not done. Examinations for the presence and number of areola/nipples were conducted on all F1 and F2 male pups on PND 13.

All F1 offspring from Cohorts 1A, 1B, and 1C were assessed for sexual maturation landmarks. Female pups were assessed daily for vaginal patency beginning on PND 28 by visual inspection of the vaginal area. Male pups were assessed daily for preputial skinfold cleavage beginning on PND 38 by visual inspection of the penis. Body weights were recorded on the day of acquisition of these developmental landmarks.

TABLE 2. F1/F2 litter observations. [a]

Observation	Time of observation (Postnatal day [PND])						
	PND 0	PND 1	PND 4 (pre-culling)	PND 7	PND 13	PND 14	PND 21
Number of live/dead pups [b]	X	X	X	X	X	X	X
Pup weight		X	X	X		X	X
Sex of each pup (M/F)		X	X				
Anogenital distance		X					
Areola/nipple retention (M)					X		

a Data were obtained from pages 35-36 of the study report.
b All pups were examined at least twice daily for mortality.

3. **Hematology and clinical chemistry:** Blood samples were collected from fasted P and F1 Cohort 1A

animals (10 animals/sex) on the day of necropsy (PND 85-93) via retro-orbital sinus puncture under isoflurane anesthesia. Additional samples were collected from some animals, as needed, due to clotting of non-serum samples. Blood smears were prepared but were not evaluated. The following CHECKED (X) parameters were examined.

a. Hematology:

X	Hematocrit (HCT) *	X	Leukocyte differential count *
X	Hemoglobin (HGB) *	X	Mean corpuscular HGB (MCH)
X	Leukocyte count (WBC) *	X	Mean corpuscular HGB concentration (MCHC)
X	Erythrocyte count (RBC) *	X	Mean corpuscular volume (MCV)
X	Platelet count *	X	Reticulocyte count
	Blood clotting measurements*		Red cell volume distribution width (RDW)
X	(Activated partial thromboplastin time, APTT)	X	Large unstained cells
	(Thromboplastin time)		Blood smear
X	(Prothrombin time)		
	(Fibrinogen concentration)		

* Recommended for extended one-generation reproductive toxicity studies based on OECD Guideline 443 (Not required for reproduction and fertility effects studies based on Guideline 870.3800).

b. Clinical chemistry:

	ELECTROLYTES		**OTHER**
X	Calcium	X	Albumin*
X	Chloride	X	Creatinine*
	Magnesium	X	Urea*
X	Phosphorus	X	Total cholesterol*
X	Potassium	X	Globulins
X	Sodium	X	Glucose*
	ENZYMES (at least 2 hepatic enzymes*)	X	Total bilirubin
X	Alkaline phosphatase (ALK)	X	Total protein*
	Cholinesterase (ChE)	X	Triglycerides
	Creatine phosphokinase	X	Albumin/Globulin ratio
	Lactic acid dehydrogenase (LDH)	X	Bile acids
X	Alanine aminotransferase (ALT/also SGPT)		
X	Aspartate aminotransferase (AST/also SGOT)		
	Sorbitol dehydrogenase		
	Gamma glutamyl transferase (GGT)		
	Glutamate dehydrogenase		

* Recommended for extended one-generation reproductive toxicity studies based on OECD Guideline 443 (Not required for reproduction and fertility effects studies based on Guideline 870.3800).

4. Hormone analysis: Blood samples were collected from the designated P and F1 Cohort 1A animals as described above. Additional samples were collected from unfasted surplus F1 weanlings (10 animals/sex/group) by aorta puncture under isoflurane anesthesia and from culled F1 animals on PND 4 (1 pup/sex/litter, if possible) via decapitation. Blood from PND 4 pups was pooled for each litter; if the target volume of 0.4 mL could not be reached, extra pups were sampled, if available, or if only a single pup was culled, as much blood as possible was collected. Serum samples were stored at −70 to −90°C until analysis for total thyroxine (T4) and/or thyroid stimulating hormone (TSH) with the Immulite 1000® Immunoassay System.

5. Urinalysis: P and F1 Cohort 1A animals were placed in metabolism cages for approximately 16 hours. The animals were deprived of food and drinking water but administered an approximately 20-mL/kg portion of filtered water. The following CHECKED (X) parameters were examined.

X	Appearance*	X	Glucose*
X	Volume*	X	Ketones
X	Specific gravity / osmolality*	X	Bilirubin (bile pigments)
X	pH*	X	Blood / blood cells*
X	Sediment (microscopic)		Potassium
X	Protein*		Chloride
	Sodium	X	Urobilinogen
X	Casts	X	Crystals
X	Epithelial cells	X	Bacteria

* Recommended for extended one-generation reproductive toxicity studies based on OECD Guideline 443 (Not required for reproduction and fertility effects studies based on Guideline 870.3800).

6. Postmortem observations:

a. P and F1 Cohort 1A adults: Moribund animals were necropsied after euthanasia by carbon dioxide inhalation and exsanguination. All surviving P and F1 Cohort 1A animals were weighed at termination after an overnight fast and subjected to a full gross necropsy after euthanasia by carbon dioxide inhalation with subsequent exsanguination. Scheduled terminations occurred after weaning of the F1 animals and confirmation that no further mating was required (P males: after a minimum of 12 weeks of dosing; P females: LD 22-24; F1 Cohort 1A: PND 85-93) or if no offspring were delivered/survived. Necropsy included an examination of the external surface, all orifices, all body cavities, and the carcass, with special attention to the reproductive system organs. For P generation females, the numbers of former implantation sites were recorded for each mated female. All uteri were stained with an ammonium sulfide solution to detect implantation sites, and the numbers of corpora lutea were recorded (but not reported). The following tissues from all P and F1 Cohort A animals were collected (X) and weighed (XX).

	DIGESTIVE SYSTEM		CARDIOVASC./HEMAT.		NEUROLOGIC
X	Tongue		Aorta	XX	Brain * +
X	Salivary glands	XX	Heart * +	X	Peripheral nerve (sciatic & tibial) *
X	Esophagus *	X [c]	Bone (sternum) with marrow *	X	Spinal cord (3 levels) *
X	Stomach *	XX [d]	Lymph nodes (mandibular, mesenteric) * +	XX	Pituitary * +
X	Duodenum *	XX	Spleen * +	X	Eyes *
X	Jejunum *	XX	Thymus * +	X	Optic nerves *
X	Ileum *				**GLANDULAR**
X	Cecum *		**UROGENITAL**	XX	Adrenal gland * +
X	Colon *	XX	Kidneys * +		Lacrimal gland
X	Rectum *	X	Urinary bladder *	XX	Thyroid (w/parathyroids) * +
XX	Liver * +	XX	Testes * +		
XX [a]	Pancreas	XX	Prostate * +		**OTHER**
		XX	Ovaries (w/oviducts) * +	X	All gross lesions/masses *
	RESPIRATORY	XX	Uterus (w/cervix) * +		Skeletal muscle *
X	Trachea *	X	Mammary gland *		Skin
X	Lungs *	XX	Epididymides/cauda * +		
X [b]	Nasopharynx	XX	Seminal vesicles (w/coagulating glands) * +		
X [b]	Larynx	X	Vagina *		
		X	Vas deferens *		

* Recommended for extended one-generation reproductive toxicity studies based on OECD Guideline 443 (lymph nodes pertain only to Cohort 1A).

\+ Organ weight determination recommended for extended one-generation reproductive toxicity studies based on OECD Guideline 443.

a Pancreas was collected from P animals only, and was weighed but not examined.

b Collected but not examined.

c Examination limited to F1 generation (10 animals/sex/group).

d Lymph nodes were collected from all animals and weighed and examined for F1 Cohort 1A animals only. Left mandibular lymph nodes were examined.

Paired organs were weighed together, except for the epididymis; if a difference in size was observed, the abnormally-sized organs were also weighed separately. Tissues were preserved in 10% neutral-buffered formalin, except the testes and epididymides that were fixed in modified Davidson's fluid and the eyes and optic nerves that were fixed in Davidson's fluid. Tissues were processed routinely and stained with hematoxylin and eosin (HE) for microscopic examination. Examinations were performed on P and F1 Cohort 1A tissues/organs from the control and high-concentration animals and on target organs from intermediate groups (P: thyroid gland; F1 Cohort 1A: thyroid gland, liver, bone marrow [first 10 animals/sex/group]). In the P generation, microscopic examinations were performed on tissues/organs from unscheduled adult deaths, on macroscopic lesions, and on reproductive organs from males that failed to sire and females that failed to deliver or had total litter loss. Additionally, the following evaluations were performed for the F1 Cohort 1A groups. The lymph nodes from the first 10 F1 Cohort 1A animals/sex/group were weighed and examined microscopically. After determination of spleen weight, half of the spleen from each of 10 animals/sex/group was used for splenic lymphocyte subpopulation analysis by flow cytometry and the other half was used for microscopic evaluation. The whole spleen was used for microscopic examination from the remaining 10 animals/sex/group.

HE-stained step sections of ovaries and corpora lutea from all F1 Cohort 1A females were prepared for quantitative examination of follicles, and evaluations were conducted in the control and high-dose groups. Detailed qualitative examinations of the testes from all F1 Cohort 1A males were also conducted. In addition to the HE-stained slide, an additional slide was prepared, stained with Periodic Acid Schiff (PAS), and examined.

Sperm analyses were performed for all surviving P and F1 Cohort 1A males. The left cauda epididymis from each male was sampled, weighed, and assessed for caudal sperm reserves and sperm motility, including progressive motility, as well as for sperm morphology (at least 200 sperm per sample, if possible).

b. **F1 Cohort 1B adults:** After mating (males) or on LDs 21-23 (females), all surviving, unfasted F1 Cohort 1B animals were weighed, euthanized by carbon dioxide inhalation with subsequent exsanguination, and subjected to a limited examination with special attention to the reproductive organs. The following tissues from all P and F1 Cohort A animals were collected (X) and weighed (XX). Microscopic examinations were not conducted.

	DIGESTIVE SYSTEM		CARDIOVASC./HEMAT.		NEUROLOGIC
	Tongue		Aorta	XX	Brain
	Salivary glands	XX [a]	Heart	X [a]	Peripheral nerve (sciatic & tibial)
	Esophagus		Bone marrow (sternum)		Spinal cord (3 levels)
	Stomach (fore and glandular)		Lymph nodes (mandibular, mesenteric)	XX	Pituitary
	Duodenum	XX	Spleen [a]		Eyes (w/ optic nerves)
	Jejunum (w/ Peyer's patches)	XX [a]	Thymus		**GLANDULAR**
	Ileum		**UROGENITAL**	XX	Adrenal gland
	Cecum	XX [a]	Kidneys		Lacrimal gland
	Colon		Urinary bladder	XX	Thyroid w/ parathyroids
	Rectum	XX	Testes		
XX [a]	Liver	XX	Prostate		**OTHER**
	Pancreas	XX [b]	Ovaries		Skeletal muscle
	RESPIRATORY	XX	Uterus w/ cervix		Skin
	Trachea		Mammary gland	X	All gross lesions/masses
	Lungs	XX	Epididymides/cauda		
	Pharynx	XX	Seminal vesicles w/ coagulating glands		
	Larynx	X	Vagina		
			Vas deferens		
		XX [b]	Oviducts		

a Weighed but not fixed.
b Ovaries and oviducts were weighed together; oviducts were then retained but not processed.

c. **F1 Cohort 1C young adults:** Scheduled necropsies of Cohort 1C animals were conducted after positive determination of vaginal patency or preputial separation. Animals were not fasted or weighed prior to termination by carbon dioxide inhalation and exsanguination. All animals were subjected to a limited examination, with special attention to the reproductive organs.

Descriptions of all macroscopic abnormalities were recorded. In case of macroscopic abnormalities, gross lesions were preserved in the most appropriate fixative.

d. F1 and F2 offspring: Scheduled necropsy of unfasted F1 surplus animals (not selected for Cohorts 1A, 1B, or 1C) was conducted on PND 21. Terminal body weight was recorded and blood samples were collected by aortic puncture under anesthesia with isoflurane from 10 animals/sex/group; brain, liver, thyroid and parathyroid glands, spleen, and thymus were weighed, and macroscopic lesions and mammary glands were collected. No microscopic examinations were conducted. The remainder of the surplus animals were euthanized via carbon dioxide inhalation and exsanguination. Animals were examined externally and sexed, with particular attention to the external reproductive genitals. Descriptions of all external abnormalities were recorded but no tissues/organs were collected.

Scheduled terminal necropsy of unfasted F2 offspring was conducted on PNDs 21-23 by intraperitoneal injection of sodium pentobarbital. All animals were subjected to limited examinations with special attention to the reproductive organs. Descriptions of all macroscopic abnormalities were recorded. The following organs were weighed from 10 animals/sex/group: brain, liver, thyroid and parathyroid glands, spleen, and thymus, and macroscopic lesions and mammary glands were collected. No microscopic examinations were conducted.

Pups culled on PND 4, and any moribund pups, were euthanized by intraperitoneal injection of sodium pentobarbitone. Euthanized pups, and any that were found dead, were necropsied. For pups found dead or euthanized moribund, the stomach was examined for the presence of milk, and defects or cause of death was evaluated, if possible. Each pup was sexed and examined for external defects with special attention to the external reproductive organs.

D. DATA ANALYSIS:

1. Statistics: Data were processed, and mean and standard deviation (SD) values were determined, as appropriate, with the litter as the basic unit. The best transformation for the data (none, log, or rank) was determined. Non- or log-transformed data were analyzed by parametric methods and rank-transformed data were analyzed by non-parametric methods.

Data were analyzed by Williams test (for parametric data) or Shirley's test (for non-parametric data). Homogeneity of means was assessed with an analysis of variances (ANOVA) for parametric data or the Kruskal-Wallis test for non-parametric data. If no trend was found and means were not homogeneous, the data were analyzed with a parametric or non-parametric Dunnett's test to assess significant differences from control. Selected incidence data were analyzed by using the Provantis data acquisition system and/or a SAS software package. A chi-squared test was used for all groups prior to a Fisher's two-tailed test with Bonferroni correction for each treated group vs. control if the chi-squared result was significant.

Estrous cycles, anogenital distance, sperm analyses, and precoital interval data were analyzed with a SAS software package. Levene's test was used to assess variance across groups and Shapiro-Wilk's test was used to assess the normality of the data distribution in each group. Data

with homogeneous variances and a normal distribution for all groups were analyzed with ANOVA followed by Dunnett's test, if significant. Data with non-homogeneous variances, or a non-normal distribution in at least one group, were analyzed with the Kruskal-Wallis test followed by Wilcoxon's rank sum test, if significant.

Significant differences were expressed at the 5% ($p \leq 0.05$), 1% ($p \leq 0.01$), or 0.1% ($p \leq 0.001$) level.

The Reviewers consider the statistical analyses appropriate.

2. Indices:

Reproductive indices: The following reproductive indices were calculated; mating and fertility indices were calculated separately by sex. The mating index was referred to as the copulation index in the study report and the post-implantation loss index was referred to as pre-birth loss; both indices were renamed for consistency with other Data Evaluation Reports.

$$\text{Mating index (\%)} = \frac{\text{\# males or females with confirmed mating}}{\text{\# males or females paired}} \times 100$$

$$\text{Fertility index (\%)} = \frac{\text{\# proven fertile males or pregnant females}}{\text{\# males or females with confirmed mating}} \times 100$$

$$\text{Precoital interval (days)} = \frac{\text{Sum of days until successful mating}}{\text{\# females with confirmed mating}} \times 100$$

$$\text{Gestation index (\%, Reviewer calculated)} = \frac{\text{\# live litters born}}{\text{\# pregnant females}} \times 100$$

$$\text{Postimplantation loss (\%)} = \frac{\text{\# of implantations} - \text{\# pups born}}{\text{\# of implantations}} \times 100$$

$$\text{Live birth index (\%)} = \frac{\text{\# of pups born alive}}{\text{\# of pups born}} \times 100$$

Offspring indices: The following offspring indices were calculated:

$$\text{Viability index (\%)} = \frac{\text{\# of live pups on PND 4 (precull)}}{\text{\# of live pups at birth}} \times 100$$

$$\text{Lactation index (\%)} = \frac{\text{\# live pups on PND 21}}{\text{\# of live pups on PND 4 (postcull)}} \times 100$$

$$\text{Sex ratio (\%M)} = \frac{\text{\# male pups}}{\text{\# of pups}} \times 100$$

$$\text{Anogenital index (mm/kg)} = \frac{\text{anogenital distance}}{3\sqrt{\text{body weight}}}$$

Estrous cycle irregularity index: The following cycle irregularity index was calculated:

$$\text{Estrous cycle irregularity index (\%)} = \frac{\text{mean SD of the length of the estrous cycle}}{\sqrt{\text{estrous cycle length}}} \times 100$$

3. **Historical control data:** Historical control data from Wistar rats were provided as follows. Data from individual studies were provided, unless indicated otherwise:
   - Maternal (gestation/lactation) body weight and food consumption (15-29 studies);
   - Parental body weight from treatment weeks 0 to 34 (aggregate form);
   - Estrous cycle data (4 studies, before treatment; 10 studies, premating treatment period);
   - Sperm data (4-12 studies);
   - Pup body weight (15 studies), plus aggregated postnatal body weight from PND 1 to 209 (females) or 258 (males);
   - Sexual maturation parameters (7 studies plus aggregated data);
   - Reproduction/litter data (15 studies);
   - AGD parameters (13 studies);
   - Selected hematology/clinical chemistry and urinalysis parameter data (aggregated form at different ages); and
   - Selected immunology data (2 studies).

   The study dates were redacted; however, for at least some parameters it appeared that the search criteria limited the provided data to studies from 2017 onwards.

II. RESULTS:

A. PARENTAL/ADULT ANIMALS:

1. Mortality and clinical signs:

a. Mortality: There were no treatment-related deaths in the P or F1 (Cohorts 1A/1B/1C) generations. In the P generation, one low-concentration female was euthanized with its litter on LD 3; this female was thin and had piloerection and all pups were thin with no milk noted in their stomachs at necropsy. Additionally, one mid-concentration female had total litter loss of one pup and was subsequently terminated and necropsied on LD 5. No mortality was noted at the high dose in the P generation or at any dose in the F1 generation.

b. Clinical signs of toxicity: There were no adverse treatment-related clinical signs in the P or F1 (Cohorts 1A/1B/1C) generations. All observed clinical signs were unrelated to treatment level occurred or in single animals in the control and/or treated groups.

2. Body weight and food consumption:

a. Males: Selected mean body weight, body weight gain, and food consumption data from P and F1 males during the pre- and post-mating intervals are presented in Table 3a.

P generation: There were no adverse treatment-related effects on body weight, body weight gain, or food consumption in the P males at any treatment level. At 1650 ppm, body weight was decreased ($p<0.001$) by 10% on Day 15 only. Overall body weight gain (Days 1-126) was also slightly decreased (not significant [NS]) with no related changes in food consumption.

F1 generation: Treatment-related decreases in body weight, body weight gain, and food consumption were noted in F1 males of all treated groups and these differences were considered adverse at ≥825 ppm. Body weights were decreased ($p<0.05$) in all treated groups in a dose-dependent manner, with decreases ranging from 4-8%, 6-11%, and 10-18% at 470, 825, and 1650 ppm, respectively. These body weight decreases were a continuation of decreases observed during the postnatal period that generally decreased further post-weaning. Similarly, body weight gain and food consumption were generally decreased ($p<0.05$) during most reported intervals. Overall body weight gains (Days 1-120) were decreased ($p<0.01$) by 8%, 12%, and 18% at 470, 825, and 1650 ppm, respectively, and overall food consumption (Days 1-72) was decreased ($p<0.01$) by 8%, 12%, and 18% at 470, 825, and 1650 ppm, respectively .

TABLE 3a. Mean (± SD) body weight and body weight gain (g) and food consumption (g/animal/day) in P and F1 males during premating. [a]

Observation/Study interval		Nominal dietary concentration (ppm)			
		0	470	825	1650
P Males (n = 25 animals / 5 cages)					
Body weight	Day 1	170.14 ± 13.21	169.91 ± 11.61	168.36 ± 10.93	168.36 ± 12.46
	Day 15	257.18 ± 21.03	254.64 ± 19.04	251.80 ± 19.58	230.59 ± 25.16*** (↓10)
	Day 36	341.71 ± 34.87	335.97 ± 29.54	333.52 ± 34.88	327.87 ± 30.22
	Day 71	413.17 ± 45.24	406.80 ± 41.59	402.73 ± 48.27	394.65 ± 35.83
	Day 99	438.59 ± 48.90	433.66 ± 48.04	424.08 ± 50.79	418.60 ± 38.18
	Day 126	465.17 ± 53.97	461.15 ± 49.85	451.10 ± 52.83	446.61 ± 39.71
Body weight gain	Days 1-8	44.24 ± 5.38	42.54 ± 4.93	42.13 ± 5.67	40.99 ± 5.83* (↓7)
	Days 8-15	42.80 ± 8.38	42.20 ± 7.68	41.31 ± 7.30	21.24 ± 15.86*** (↓50)
	Days 15-22	35.58 ± 7.17	34.96 ± 6.76	32.35 ± 6.94	52.88 ± 17.90** (↑49)
	Days 29-36	24.29 ± 5.61	20.96 ± 4.84	23.56 ± 6.00	19.86 ± 5.71**(↓18)
	Days 64-71	14.04 ± 4.85	14.87 ± 4.16	14.37 ± 4.80	13.67 ± 3.80
	Days 99-106	13.45 ± 6.13	7.63 ± 7.07** (↓43)	10.02 ± 3.90** (↓25)	9.09 ± 3.71** (↓32)
	Days 120-126	4.56 ± 4.21	8.67 ± 3.29* (↑90)	5.54 ± 5.70* (↑22)	7.89 ± 2.45** (↑73)
	Days 1-126	295.02 ± 49.49	291.24 ± 44.06	282.74 ± 46.75	278.26 ± 33.87 (↓6)
Food consumption	Days 1-71	22.41 ± 1.15	22.28 ± 1.18	22.31 ± 0.94	22.34 ± 0.46 [b]
F1 Males- Cohorts 1A, 1B, and /or 1C (n =20- 60 animals / 4-12 cages) [c]					
Body weight	Day 1	70.23 ± 9.12	67.02 ± 7.60* (↓5)	65.76 ± 8.14** (↓6)	62.50 ± 7.35*** (↓11)
	Day 36	289.28 ± 23.24	270.68 ± 23.66*** (↓6)	268.97 ± 23.50*** (↓7)	255.62 ± 25.06*** (↓12)
	Day 72	406.12 ± 40.18	375.13 ± 34.65** (↓8)	364.09 ± 43.97*** (↓10)	331.80 ± 28.76*** (↓18)
	Day 99	448.23 ± 47.60	415.58 ± 40.07* (↓7)	401.42 ± 51.22*** (↓10)	374.45 ± 32.61*** (↓16)
	Day 120	478.57 ± 52.18	441.16 ± 42.91** (↓8)	425.46 ± 52.57*** (↓11)	396.27 ± 32.85*** (↓17)
Body weight gain	Days 1-8	43.43 ± 5.43	41.95 ± 4.09	40.81 ± 4.30** (↓6)	39.39 ± 4.89*** (↓9)
	Days 29-36	35.55 ± 6.44	33.39 ± 8.21	33.62 ± 6.61	30.99 ± 6.14*** (↓13)
	Days 1-57	290.56 ± 32.50	266.37 ± 37.46*** (↓8)	267.30 ± 28.98*** (↓8)	250.82 ± 30.53*** (↓14)
	Days 64-72	13.66 ± 3.33	18.22 ± 12.10	15.21 ± 2.79	12.84 ± 3.92
	Days 1-72	334.81 ± 36.51	308.38 ± 34.84** (↓8)	297.90 ± 39.93*** (↓11)	270.97 ± 25.47*** (↓19)
	Days 99-106	11.24 ± 3.99	7.96 ± 4.55** (↓29)	5.47 ± 2.76*** (↓51)	7.04 ± 3.45*** (↓37)
	Days 113-120	8.98 ± 3.24	10.03 ± 3.51	9.43 ± 4.35	8.00 ± 4.17
	Days 1-120	407.26 ± 48.80	374.41 ± 41.90** (↓8)	359.27 ± 48.51*** (↓12)	335.44 ± 29.96*** (↓18)
Food consumption	Days 1-4	12.56 ± 0.56	12.56 ± 0.64	12.69 ± 1.83	12.18 ± 0.51** (↓3)
	Days 32-36	24.56 ± 1.10	23.39 ± 1.29*** (↓5)	23.45 ± 1.65*** (↓5)	23.23 ± 0.94*** (↓6)
	Days 1-57	21.74 ± 0.82	20.77 ± 1.11*** (↓4)	20.61 ± 1.38*** (↓5)	20.29 ± 0.66*** (↓7)
	Days 68-72	24.15 ± 0.36	22.98 ± 1.32** (↓5)	22.98 ± 1.99** (↓5)	21.65 ± 1.19*** (↓10)
	Days 1-72	22.84 ± 0.33	21.61 ± 1.02*** (↓5)	21.26 ± 1.48*** (↓7)	20.63 ± 0.58*** (↓10)

a Data were obtained from Tables 3-4, 11, 12, 26-27, and 34 on pages 125-134, 149-154, 205-215, and 233-238 of the study report. Percentage differences from control are presented in parentheses.

b Data excluded from one cage.

c Group sizes were as follows: on Days 1-57, 60 animals and 12 cages (all cohorts), on Days 57-64, 40 animals and 8 cages (Cohorts 1A and 1B), and on Days 64-120, 20 animals and 4-5 cages (Cohort 1B).

* Significantly different from control; p<0.05.

** Significantly different from control; p<0.01.

*** Significantly different from control; p<0.001.

b. Females:

1. Premating: Selected mean body weight, body weight gain, and food consumption data during premating in P and F1 females are presented in Table 3b.

P generation: There were no treatment-related effects on body weight, body weight gain, or food consumption in the P females at any treatment level.

F1 generation: Treatment-related decreases in body weight, body weight gain, and food consumption were noted in the F1 ≥825 ppm females Body weights were decreased ($p<0.05$) in a dose-dependent manner by 3-5% and 4-9% in the 825 and 1650 ppm, respectively. These body weight decreases were a continuation of decreases observed during the postnatal period. Overall body weight gains were decreased ($p<0.05$) at all dietary concentrations during Days 1-57 and at 1650 ppm during Days 1-72 Overall food consumption during Days 1-57 and 1-72 was decreased ($p<0.01$) by 2% each and 4-5% at 825 and 1650 ppm, respectively. There were no treatment-related effects on body eight, body weight gain, or food consumption at 470 ppm.

TABLE 3b. Mean (± SD) body weight and body weight gain (g) and food consumption (g/animal/day) in P and F1 females during premating. [a]

Observation /Study interval		Nominal dietary concentration (ppm)			
		0	470	825	1650
P Females (n = 25 animals and 5 cages)					
Body weight	Day 1	133.47 ± 10.92	127.72 ± 8.81	132.72 ± 10.67	131.33 ± 9.87
	Day 36	212.61 ± 18.61	206.57 ± 17.47	211.26 ± 16.60	205.83 ± 12.34
	Day 71	246.50 ± 19.33	238.30 ± 21.71	244.90 ± 20.95	238.18 ± 17.61
Body weight gain	Days 1-8	20.31 ± 3.22	21.94 ± 4.23	21.25 ± 3.92	20.48 ± 3.67
	Days 22-29	14.07 ± 11.82	10.25 ± 3.69	12.20 ± 10.94	8.60 ± 5.62** (↓39)
	Days 29-36	9.44 ± 10.25	10.63 ± 4.76	9.70 ± 11.52	11.93 ± 5.03 (↑26)
	Days 64-71	7.03 ± 5.32	5.26 ± 5.87	7.54 ± 5.92	6.61 ± 5.72
	Days 1-71	113.03 ± 13.29	110.57 ± 17.79	112.18 ± 14.68	106.85 ± 13.96
Food consumption	Days 1-4	13.84 ± 0.71	13.44 ± 0.75	13.81 ± 0.41	13.63 ± 0.46
	Days 32-36	16.76 ± 1.06	16.94 ± 1.25	17.04 ± 0.92	16.23 ± 0.79
	Days 67-71	16.75 ± 0.77	16.50 ± 1.39	16.80 ± 0.77	15.92 ± 0.64** (↓5)
	Days 1-71	15.99 ± 0.82	16.03 ± 1.03	16.32 ± 0.51	15.70 ± 0.58
F1 Females-Cohorts 1A, 1B, and/or 1C (n = 20-60 animals and 4-12 cages) [b]					
Body weight	Day 1	65.29 ± 8.28 [c]	63.48 ± 7.21	62.32 ± 7.47* (↓5)	60.85 ± 6.64** (↓7)
	Day 36	187.05 ± 15.37	181.48 ± 15.96* (↓3)	179.32 ± 14.02** (↓4)	176.73 ± 13.63*** (↓6)
	Day 72	234.11 ± 21.62	223.33 ± 23.74	221.56 ± 20.25* (↓5)	213.16 ± 16.56** (↓9)
Body weight gain	Days 1-8	35.36 ± 4.20 [c]	34.72 ± 3.55	34.46 ± 3.85	34.24 ± 3.71
	Days 29-36	14.92 ± 4.61	13.98 ± 4.42	13.84 ± 4.24	12.75 ± 4.36** (↓15)
	Day 1-57	150.36 ± 14.67	144.55 ± 18.80* (↓4)	143.94 ± 12.76* (↓4)	142.15 ± 12.18** (↓5)
	Days 64-72	7.27 ± 3.58	6.70 ± 3.73	7.00 ± 2.23	4.45 ± 2.39** (↓39)
	Days 1-72	167.40 ± 17.24	159.53 ± 22.25	158.44 ± 14.59	152.85 ± 11.59** (↓9)
Food consumption	Days 1-4	10.94 ± 0.65	11.15 ± 0.56	10.69 ± 0.47** (↓2)	10.76 ± 0.67** (↓2)
	Days 32-36	16.34 ± 0.74	16.34 ± 0.79	15.78 ± 0.86*** (↓3)	15.66 ± 0.72*** (↓4)
	Days 1-57	15.23 ± 1.29	15.43 ± 0.72	14.89 ± 0.65*** (↓2)	14.98 ± 0.60*** (↓2)
	Days 68-72	17.64 ± 0.55	17.41 ± 0.46	16.48 ± 0.74*** (↓7)	15.89 ± 0.52*** (↓10)
	Days 1-72	16.10 ± 0.40	16.10 ± 0.44	15.45 ± 0.70** (↓4)	15.34 ± 0.62*** (↓5)

a Data were obtained from Tables 5-6, 12, 28, 29, and 35 on pages 135-140, 155-160, 216-222, and 239-244 of the study report. Percentage differences from control are presented in parentheses.

b Group sizes were as follows: on Days 1-57, 60 animals and 12 cages (all cohorts), on Days 57-64, 40 animals and 8 cages (Cohorts 1A and 1B), and on Days 64-72, 20 animals and 4-5 cages (Cohort 1B).

c n = 59 animals in control group on Day 1 only.

* Significantly different from control; p<0.05.

** Significantly different from control; p<0.01.

*** Significantly different from control; p<0.001.

2. <u>Gestation</u>: Selected body weight, body weight gain, and food consumption data in the P and F1 Cohort 1B females during gestation are presented in Table 3c.

<u>P generation</u>: In the P animals, the interval body weights did not demonstrate a clear dose response, as body weights at 470 and 825 ppm were both slightly decreased (NS) in a similar manner relative to control, with decreases (generally p<0.05) of 4-6% at 1650 ppm. Overall body weight gain (GDs 0-20) was decreased (p<0.05) by 9%. These changes, and the decreased () body weight gains during GDs 0-4 at ≥825 ppm were considered potentially related to treatment, but non-adverse. In addition, there were treatment-related effects on body weight, body weight gain, or food consumption at 470 ppm.

F1 generation: Generally dose-dependent differences in body weight (except ≤825 ppm), body weight gain, and food consumption were observed in all treatment groups; these effects were considered adverse at 1650 ppm only based on body weight decreases ($p<0.01$) of 9-11%, body weight gain decreases ($p<0.05$) of 36% and 16% during GDs 4-7 and 0-20, respectively, and an overall food consumption decrease ($p<0.001$) of 12% during GDs 0-20.

TABLE 3c. Mean (± SD) body weight and body weight gain (g) and food consumption (g/animal/day) in P and F1 Cohort 1B females during gestation. [a]

Observation /Study interval		Nominal dietary concentration (ppm)			
		0	470	825	1650
P Females (n = 25, 24, 25, and 25, respectively)					
Body weight	GD 0	245.80 ± 19.92	237.57 ± 20.28	243.12 ± 20.33	236.49 ± 17.04
	GD 4	261.96 ± 20.62	251.85 ± 20.33	255.79 ± 21.49	248.63 ± 18.23* (↓5)
	GD 7	269.06 ± 19.37	258.72 ± 20.56	261.48 ± 22.06	254.65 ± 19.30* (↓5)
	GD 14	295.17 ± 21.39	281.79 ± 21.98	286.26 ± 23.86	277.45 ± 21.59** (↓6)
	GD 20	352.81 ± 28.67	340.70 ± 27.38	344.76 ± 28.65	334.17 ± 28.58* (↓5)
Body weight gain	GD 0-4	16.16 ± 4.86	14.28 ± 4.31	12.67 ± 4.24** (↓22)	12.14 ± 4.53** (↓25)
	GD 4-7	7.10 ± 3.52	6.86 ± 3.81	5.69 ± 4.26	6.02 ± 3.18
	GD 7-14 [b]	26.11	23.07	24.77	22.80
	GD 14-20 [b]	57.64	58.91	58.29	56.72
	GD 0-20	107.01 ± 17.04	103.13 ± 14.66	101.63 ± 13.53	97.68 ± 14.82* (↓9)
Food consumption	GD 0-20	20.87 ± 1.87	20.72 ± 2.00 [c]	20.48 ± 1.90	20.00 ± 2.23 [c]
F1 Females- Cohort 1B (n = 17, 19, 20, and 19-20, respectively)					
Body weight	GD 0	234.67 ± 23.24	220.22 ± 22.26* (↓6)	221.75 ± 20.48* (↓6)	214.47 ± 14.58** (↓9)
	GD 7	258.17 ± 25.29	243.42 ± 23.79* (↓6)	241.91 ± 24.25* (↓6)	231.33 ± 14.30*** (↓10)
	GD 14	284.61 ± 27.59	268.81 ± 23.34* (↓6)	265.88 ± 28.70* (↓7)	254.04 ± 17.40*** (↓11)
	GD 20	341.92 ± 36.09	325.13 ± 27.74 (↓5)	320.71 ± 40.39* (↓6)	304.62 ± 29.12** (↓11)
Body weight gain	GD 0-4	15.32 ± 4.53	15.66 ± 5.59	13.34 ± 6.11	11.66 ± 5.08
	GD 4-7	8.18 ± 3.94	7.54 ± 3.59	6.83 ± 2.99	5.19 ± 3.41* (↓36)
	GD 7-14 [b]	26.44	25.39	23.97	22.71
	GD 14-20 [b]	57.31	56.32	54.88	50.58
	GD 0-20	107.25 ± 21.91	104.92 ± 15.28 (↓2)	98.96 ± 27.67 (↓8)	90.15 ± 23.01* (↓16)
Food consumption	GD 0-20	21.40 ± 2.19	20.13 ± 1.59* (↓6)	19.88 ± 2.15* (↓7) [c]	18.92 ± 1.58*** (↓12) [c]

a Data were obtained from Tables 7-8, 13, 30-31, and 36 on pages 141-144, 161-162, 223-228, and 245-246 of the study report. Percentage differences from control are presented in parentheses.
b Calculated by the Reviewers from group means.
c Data excluded from 1-2 animals.
* Significantly different from control; $p<0.05$.
** Significantly different from control; $p<0.01$.
*** Significantly different from control; $p<0.001$.

3. Lactation: Selected body weight, body weight gain, and food consumption data in the P and F1 Cohort 1B females during lactation are presented in Table 3d. There were no adverse treatment-related effects in either generation during lactation as all potentially treatment-related changes demonstrated recovery throughout the period.

P generation: Body weight was decreased ($p<0.05$) by 5-7% in the high-concentration treatment group (730-1200 ppm) on LDs 1 and 14 and decreased ($p<0.05$) by 2-6% in all treated P females on

LD 21 (in a manner unrelated to dose). There were no treat-related effects on overall body weight gains. Food consumption was decreased ($p<0.05$) by 5-6% and 8% at 205-340 and 365-600 ppm, respectively, during LDs 10-17 and decreased ($p<0.05$) by 7-13% at 730-1200 ppm during LDs 10-21. Overall food consumption (LDs 1-21) was decreased ($p<0.05$) by 5% and 8% in the mid- and high-concentration treatment groups, respectively.

F1 generation: Although treatment-related decreases ($p<0.01$) in body weight were observed in the 730-1200 ppm females throughout lactation, these changes were attenuated by LD 21, with no treatment-related effects on overall body weight gain at any treatment level. Food consumption was decreased ($p<0.01$) by 8-12% in all treatment groups during LD 1-4, with attenuated decreases in the 730-1200 ppm animals during the remainder of lactation. Overall food consumption was decreased ($p<0.05$) by 3% in the high concentration-treatment group only.

TABLE 3d. Mean (± SD) body weight and body weight gain (g) and food consumption (g/animal/day) in P and F1 Cohort 1B females during lactation. [a]

Observation /Study interval		Nominal dietary concentration (ppm)			
		0	470 (205-340)	825 (365-600)	1650 (730-1200)
P Females (n = 25, 23, 24-25, and 24-25, respectively)					
Body weight	LD 1	266.46 ± 24.79	254.79 ± 20.42	256.04 ± 26.33	247.88 ± 24.20** (↓7)
	LD 4	278.64 ± 22.77	269.45 ± 22.37	274.20 ± 24.06	267.28 ± 22.01
	LD 7	284.91 ± 23.41	279.10 ± 21.85	284.65 ± 22.36	275.25 ± 20.50
	LD 14	303.69 ± 19.53	290.73 ± 23.55	299.96 ± 24.07	288.75 ± 21.91* (↓5)
	LD 21	299.46 ± 19.35	282.13 ± 21.14* (↓6)	294.23 ± 20.56* (↓2)	285.98 ± 22.47* (↓5)
Body weight gain	LD 1-4	12.18 ± 9.37	14.66 ± 9.57	18.15 ± 9.39* (↑49)	19.40 ± 7.22** (↑59)
	LD 4-7	6.27 ± 8.42	9.66 ± 6.66	9.15 ± 5.83	7.97 ± 6.18
	LD 7-14 [b]	18.78	11.63	15.31	13.50
	LD 14-21 [b]	−4.23	−8.60	−5.73	−2.77
	LD 1-21	33.00 ± 15.95	27.34 ± 13.90	37.33±12.88	38.09 ± 15.05
Food consumption	LD 1-4	31.96 ± 8.20	31.49 ± 4.21	28.75 ± 5.71	29.74 ± 4.17
	LD 10-14	58.53 ± 5.99 [c]	54.86 ± 4.93** (↓6)	54.01 ± 5.08** (↓8)	51.08 ± 4.92*** (↓13)
	LD 14-17	64.07 ± 6.26	60.59 ± 6.28* (↓5)	58.99 ± 7.11** (↓8)	57.60 ± 7.08*** (↓10)
	LD 1-21	53.14 ± 6.61	51.97 ± 4.09 [c]	50.72 ± 3.94* (↓5) [c]	48.98 ± 4.60*** (↓8) [c]
F1 Females- Cohort 1B (n = 19, 20, 19, and 19, respectively)					
Body weight	LD 1	261.79 ± 22.07	251.53 ± 28.41	248.03 ± 30.49	238.53 ± 19.21** (↓9)
	LD 4	277.16 ± 26.32	270.31 ± 25.80	267.12 ± 27.46	252.47 ± 17.16** (↓9)
	LD 7	283.46 ± 27.08	281.47 ± 25.43	277.46 ± 28.10	260.55 ± 17.70** (↓8)
	LD 14	296.63 ± 29.21	295.17 ± 25.82	291.44 ± 26.87	274.29 ± 16.18** (↓8)
	LD 21	291.39 ± 25.20	287.20 ± 29.52	283.94 ± 25.21	269.69 ± 17.43** (↓7)
Body weight gain	LD 1-4	15.36 ± 7.41	18.78 ± 11.21	19.09 ± 9.43	13.94 ± 6.22
	LD 4-7	6.30 ± 8.43	11.16 ± 6.65	10.34 ± 6.63	8.08 ± 4.88
	LD 7-14 [b]	13.17	13.71	13.98	13.74
	LD 14-21 [b]	−5.24	−7.97	−7.50	−4.60
	LD 1-21	29.60 ± 14.42	35.68 ± 15.51	35.91 ± 11.85	31.16 ± 13.60
Food consumption	LD 1-4	36.89 ± 9.93	34.00 ± 5.75* (↓8)	33.64 ± 4.80** (↓9)	32.46 ± 4.45** (↓12)
	LD 7-10	47.54 ± 8.99	49.60 ± 4.71	47.21 ± 5.24	45.65 ± 4.00* (↓4)
	LD 14-17	58.30 ± 10.63	57.95 ± 8.87	54.24 ± 9.98	53.98 ± 7.54* (↓7)
	LD 1-21	49.79 ± 10.28 [d]	52.01 ± 4.60	50.29 ± 4.27	48.38 ± 2.68** (↓3) [c]

a Data were obtained from Tables 9-10, 14, 32-33, and 37 on pages 145-148, 163-164, 229-232, and 247-248 of the study report. Percentage differences from control are presented in parentheses.

b Calculated by the Reviewers from group means.

c Data excluded from a single animal.

d Data excluded from four animals.

* Significantly different from control; p<0.05.

** Significantly different from control; p<0.01.

*** Significantly different from control; p<0.001.

3. <u>Test material intake</u>: The mean test material intakes for the P and F1 animals during the premating, gestation, and lactation periods are presented in Table 4.

TABLE 4. Mean test material intake (mg/kg/day) during premating, gestation, and lactation. [a]

Sex/Interval	Nominal dietary concentration (ppm)					
	470	825	1650	470	825	1650
	P			F1		
Males						
Premating	25.8	45.9	94.1	33.9	59.6	123.2
Females						
Premating	29.0	50.6	99.8	35.0	60.0	122.1
Gestation	26.8	45.6	91.7	27.4	47.6	95.2
Lactation [b]	34.4	57.5	116.3	34.4	59.9	121.0

a Data were obtained from page 26 and Text Tables 19 and 24 on pages 57 and 64 of the study report.
b During lactation, dams in the 470, 825, and 1650 ppm nominal dietary concentration groups were administered diets containing 205-340 ppm, 365-600 ppm, and 730-1200 ppm, respectively.

4. Clinical pathology:

a. Hematology: There were no adverse treatment-related effects on hematology parameters in the P or F1 Cohort 1A animals. In P animals, decreases ($p<0.05$) were observed in hemoglobin and hematocrit (1650 ppm males), absolute monocyte counts (all treated males), activated partial thromboplastin time (APTT, ≥825 ppm females), and prothrombin time (PT, 1650 ppm females). In the F1 animals, decreases ($p<0.05$) in prothrombin time (1650 ppm males) and platelets (825 ppm males) were observed, in addition to increases () in white blood cell and absolute lymphocyte counts (≥825 ppm females). These changes were considered unrelated to treatment or non-adverse due to their minimal magnitude, the absence of a dose response, occurrence in only one sex, and/or their falling within the respective HCD ranges.

b. Clinical chemistry: There were no clearly adverse treatment-related effects on clinical chemistry parameters in the P or F1 Cohort 1A animals; although numerous differences from control were noted, most mean values fell within the HCD ranges. Effects of clinical chemistry parameters included: decreased ($p<0.05$) total bilirubin r in P and F1 males (all treatment levels), ≥825 ppm P females, and 1650 ppm F1 females; increased ($p<0.01$) albumin to globulin ratio (all treated P and F1 males),; with concurrent decreases ($p<0.05$) in globulins and total protein in the P males and increased ($p<0.01$) albumin in the 1650 ppm F1 males; increased urea ($p<0.05$) (1650 ppm P and F1 males and ≥825 ppm F1 females), with a concurrent increase ($p<0.05$) in creatine 1650 ppm F1 females). Differences observed in a single sex of a single generation included: decreased ($p<0.05$) phosphorus at all treatment levels and increased ($p<0.01$) alkaline phosphatase at 1650 ppm in the P males; decreased glucose in the 1650 ppm P females; decreased ($p<0.05$) triglycerides and aspartate aminotransferase (AST) in the ≥825 ppm F1 males; and increased ($p<0.05$) ALT in the 1650 ppm F1 females.

c. Thyroid hormone analysis: The hormone data are presented in Table 5 for the P and F1 Cohort 1A animals.

Serum concentrations of T4 were decreased ($p<0.01$) by 25-26% in the ≥825 ppm P males and by 34% in the 1650 ppm F1 males. There were no treatment-related effects on TSH, although an increase (NS) of 82% was observed in the 1650 ppm F1 males.

There were no treatment-related effects on T4 (P and F1) and TSH (P) serum concentrations in females. The ≥825 ppm F1 females had increased ($p<0.05$) TSH concentrations in the absence ofT4 changes. The TSH concentration in the 1650 ppm F1 females was disproportionately influenced by a single female with an increased value; after excluding this female, the mean value was 0.22 µIU/mL. The difference in the 1650 ppm animals is difficult to interpret due to the variability, and the toxicological relevance is considered equivocal.

TABLE 5. Mean (± SD) serum hormone concentrations in P and F1 animals. [a]

Parameter	Nominal dietary concentration (ppm)			
	0	470	825	1650
P males				
T4 (µg/dL)	6.05 ± 1.07	5.38 ± 0.81	4.55 ± 0.88** (↓25)	4.45 ± 1.10** (↓26)
TSH (µIU/mL)	0.123 ± 0.098	0.140 ± 0.119	0.105 ± 0.065	0.143 ± 0.079
P females				
T4 (µg/dL)	6.84 ± 1.85	5.93 ± 1.12	5.94 ± 1.14	6.11 ± 0.62
TSH (µIU/mL)	0.175 ± 0.083	0.306 ± 0.156	0.232 ± 0.143	0.367 ± 0.260
F1 Cohort 1A males				
T4 (µg/dL)	6.65 ± 1.21	6.17 ± 0.95	5.60 ± 1.86	4.38 ± 0.68*** (↓34)
TSH (µIU/mL)	0.152 ± 0.246	0.171 ± 0.153	0.158 ± 0.143	0.276 ± 0.191 (↑82)
F1 Cohort 1A females				
T4 (µg/dL)	3.81 ± 1.20	3.77 ± 0.90	3.67 ± 1.43	3.61 ± 0.59
TSH (µIU/mL)	0.059 ± 0.079	0.057 ± 0.032	0.105 ± 0.088* (↑78)	0.808 ± 1.885 * [b]

a Data were obtained from Appendix 45 on pages 1521-1522 of the study report; n = 10. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.

b One female (#346) had an unusually increased value of 6.08 µIU/mL; if excluded, the mean ± SD (calculated by the Reviewers) was 0.223 ± 0.369 (3.8-fold greater than control).

* Significantly different from control; $p<0.05$.

** Significantly different from control; p0.01.

*** Significantly different from control; p0.001.

d. Urinalysis: There were no treatment-related effects on urinalysis parameters in the P or F1 Cohort 1A animals. The decrease ($p<0.05$) in pH in the 1650 ppm F1 females was considered incidental as this value was within the expected range.

e. Splenic lymphocyte subpopulation analysis: There were no treatment-related effects on the immunophenotyping of splenocytes in the F1 Cohort 1A animals.

5. Reproductive function:

a. Estrous cycle length and periodicity: There were no treatment-related adverse effects on the mean length and regularity of the estrous cycles of the P or F1 Cohort 1A females. The percentage of females acyclic or with "acyclic periods" was increased ($p<0.01$) in the 1650 ppm P animals (24% treated vs. 8% control). The toxicological relevance of this increase was equivocal as the percentage in the control, 470 ppm, and 825 ppm females appeared to be low relative to the percentages noted in the F1 animals (except for the decrease in the 470 ppm females).

b. Sperm measures: There were no treatment-related effects on the evaluated sperm parameters or mean cauda epididymis weights in the P and F1 Cohort 1A males.

6. Reproductive performance: Reproductive performance data for the P and F1 Cohort 1B animals are presented in Table 6. There were no treatment-related effects on reproductive performance.

TABLE 6. Reproductive performance in P and F1 Cohort 1B parental animals. [a]

Parameter	Nominal dietary concentration (ppm)			
	0	470	825	1650
P animals				
Number of males paired	25	25	25	25
Number of females paired	25	25	25	25
Number of males mating	25	25	25	25
Number of females mating	25	25	25	25
Mating index (%)	100	100	100	100
Number of females pregnant	25	24	25	25
Fertility index (%)	100	96	100	100
Pre-coital interval (days; mean ± SD)	3.0 ± 1.6	2.7 ± 1.3	2.6 ± 1.2	2.4 ± 1.1
Gestation length (days; mean ± SD)	22.3 ± 0.5	22.3 ± 0.5	22.3 ± 0.5	22.2 ± 0.4
Gestation index (%) [b]	100	96	100	100
F1 Cohort 1B animals				
Number of males paired	20	20	20	20
Number of females paired	20	20	20	20
Number of males mating	19	20	20	20
Number of females mating	19	20	20	20
Mating index (%)	95	100	100	100
Number of females pregnant	19	20	19	19
Fertility index (%)	100	100	95	95
Pre-coital interval (days; mean ± SD)	2.5 ± 1.0	2.7 ± 1.3	2.5 ± 1.2	2.6 ± 1.0
Gestation length (days; mean ± SD)	22.2 ± 0.4	22.2 ± 0.4	22.1 ± 0.3	22.1 ± 0.3
Gestation index (%) [b]	100	100	100	100

a Data were obtained or derived from Tables 19-20 and 43-44 on pages 178-179 and 267-268 of the study report.
b Calculated by the Reviewers from data in Tables 19-20 and 43-44; not subjected to statistical analysis.

7. Parental postmortem results:

a. Organ weights: Selected organ weight data from the P and F1 (Cohorts 1A/1B) animals are presented in Tables 7a and 7b, respectively. There were no treatment-related effects on terminal body weights in the P animals. Terminal body weights were decreased ($p<0.05$) in all treated F1 Cohort 1A and 1B males (7-15% and 8-17%, respectively) and the 1650 ppm F1 Cohort 1A and 1B females (6% and 8%, respectively).

Treatment-related increases in the absolute and/or relative (to body) thyroid gland weights were observed in males and females of both generations, generally in a dose-related manner, and correlated with microscopic findings of follicular hypertrophy. In the P animals, absolute thyroid weights were increased ($p<0.05$) by 14% and 24% in the 1650 ppm males and females, respectively, with relative (to body) thyroid weight increases ($p<0.05$) of 18% in the 1650 ppm males and 16-28% in all treated females. In the F1 animals, absolute thyroid weights were increased ($p<0.05$) by 15-21% in all treated Cohort 1A males, with relative (to body) thyroid weight

increases (p<0.05) of 25-44% in all Cohort 1A males, 15% in the 1650 ppm Cohort 1A females, 13-25% in the ≥825 ppm Cohort 1B males, and 24% in the 1650 ppm Cohort 1B females.

Relative (to body) liver weights were increased (p<0.05) in the F1 Cohorts 1A and 1B 1650 ppm males, with no concomitant changes in absolute liver weights. . These alterations were correlated with observations of centrilobular hypertrophy. Relative (to body) liver weights were also increased (p<0.05) by 3-16% in the ≥825 ppm P males, all treated P females, and the F1 Cohort 1B females. As these changes were of relatively low magnitude and no correlating microscopic findings, they were considered adaptive responses and non-adverse.

Absolute spleen weights were decreased (p,0.05) in all treated P and F1 Cohort 1A females, with no relationship to dose level and relative (to body) spleen weights were decreased (p<0.05) in the ≥825 ppm P and the F1 Cohort 1A females .

Other significant differences in absolute and /or relative organ weights were attributed to terminal body weight decreases, were unrelated to treatment levels, occurred in a single F1 cohort, had no correlating microscopic or clinical pathology findings, and/or were of relatively small magnitude. Therefore, they were not considered related to treatment.

TABLE 7a. Selected mean (± SD) absolute and relative (to body) organ weights in the P animals. [a]

Parameter	Nominal dietary concentration (ppm)			
	0	470	825	1650
Males (n = 25)				
Terminal BW (g)	437.31 ± 51.08	430.87 ± 45.53	423.82 ± 50.23	416.48 ± 37.24
Rel. liver (%)	3.09 ± 0.37	3.27 ± 0.46	3.32 ± 0.34* (↑7)	3.57 ± 0.47** (↑16)
Abs. thyroid (g)	0.0211 ± 0.0046	0.0213 ± 0.0047	0.0220 ± 0.0050	0.0240 ± 0.0050* (↑14)
Rel. thyroid (%)	0.00487 ± 0.00126	0.00494 ± 0.00088	0.00518 ± 0.00098	0.00575 ± 0.00101** (↑18)
Females (n = 25, 23, 24, and 25, respectively)				
Terminal BW (g)	251.41 ± 18.52	239.13 ± 19.81	250.59 ± 19.47	244.13 ± 20.42
Rel. liver (%)	4.60 ± 0.44	4.91 ± 0.40* (↑7)	4.73 ± 0.38* (↑3)	4.97 ± 0.43** (↑8)
Abs. spleen (g)	0.657 ± 0.121	0.593 ± 0.094* (↓10)	0.610 ± 0.083* (↓7)	0.577 ± 0.072** (↓12)
Rel. spleen (%)	0.261 ± 0.039	0.247 ± 0.027	0.244 ± 0.033* (↓7)	0.237 ± 0.029* (↓9)
Abs. thyroid (g)	0.0140 ± 0.0045	0.0161 ± 0.0045	0.0162 ± 0.0050	0.0173 ± 0.0037* (↑24)
Rel. thyroid (%)	0.00556 ± 0.00183	0.00676 ± 0.00192* (↑22)	0.00645 ± 0.00185* (↑16)	0.00713 ± 0.00167** (↑28)

a Data were obtained from Appendix 46 on pages 1554-1560 of the study report. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.

* Significantly different from control; p<0.05.

** Significantly different from control; p<0.01.

TABLE 7b. Selected mean (± SD) absolute and relative (to body) organ weights in the F1 animals. [a]

Parameter	Nominal dietary concentration (ppm)			
	0	470	825	1650
F1 Cohort 1A Males (n = 20)				
Terminal BW (g)	357.17 ± 44.41	331.04 ± 47.09* (↓7)	318.09 ± 23.65** (↓11)	301.87 ± 33.39* (↓15)
Rel. liver (%)	3.95 ± 0.46	4.13 ± 0.38	4.15 ± 0.61	4.74 ± 0.75** (↑20)
Abs. thyroid (g)	0.0160 ± 0.0039	0.0184 ± 0.0035* (↑15)	0.0187 ± 0.0040* (↑17)	0.0194 ± 0.0033** (↑21)
Rel. thyroid (%)	0.00450 ± 0.00103	0.00561 ± 0.00113** (↑25)	0.00584 ± 0.00100** (↑30)	0.00649 ± 0.00129** (↑44)
F1 Cohort 1A Females (n = 20)				
Terminal BW (g)	205.85 ± 12.79	195.44 ± 16.39	201.41 ± 12.62	193.55 ± 15.49** (↓6)
Abs. spleen (g)	0.508 ± 0.066	0.449 ± 0.052* (↓12)	0.495 ± 0.063* (↓3)	0.440 ± 0.055** (↓13)
Rel. spleen (%)	0.247 ±0.027	0.230±0.021	0.245 ± 0.0260	0.228 ± 0.024* (↓8)
Abs. thyroid (g)	0.0123 ± 0.0023	0.0123 ± 0.0033	0.0118 ± 0.0022	0.0133 ± 0.0021
Rel. thyroid (%)	0.00599 ± 0.00105	0.00631 ± 0.00169	0.00587 ± 0.00101	0.00690 ± 0.00107* (↑15)
F1 Cohort 1B Males (n = 20)				
Terminal BW (g)	474.80 ± 51.90	438.78 ± 43.41** (↓8)	422.34 ± 51.89** (↓11)	394.00 ± 32.85** (↓17)
Rel. liver (%)	4.46 ± 0.40	4.75 ± 0.61	4.78 ± 0.64	4.93 ± 0.72* (↑11)
Abs. thyroid (g)	0.0214 ± 0.0051	0.0202 ± 0.0049	0.0215 ± 0.0044	0.0221 ± 0.0041
Rel. thyroid (%)	0.00452 ± 0.00102	0.00460 ± 0.00093	0.00508 ± 0.00081* (↑12)	0.00564 ± 0.00101** (↑25)
F1 Cohort 1B Females (n = 19, 20, 19, and 19, respectively)				
Terminal BW (g)	270.44 ± 22.70	265.35 ± 27.02	263.18 ± 23.60	248.59 ± 16.45** (↓8)
Rel. liver (%)	5.15 ± 0.58	5.42 ± 0.42	5.36 ± 0.50	5.63 ± 0.35** (↑9)
Abs. thyroid (g)	0.0128 ± 0.0026	0.0129 ± 0.0036	0.0142 ± 0.0035	0.0145 ± 0.0026
Rel. thyroid (%)	0.00474 ± 0.00087	0.00492 ± 0.00138	0.00541 ± 0.00137	0.00586 ± 0.00110** (↑24)

a Data were obtained from Appendix 46 on pages 1605-1611 and 1631-1637 of the study report. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.

* Significantly different from control; $p<0.05$.

** Significantly different from control; $p<0.01$.

b. Pathology:

1) Macroscopic examination: There were no treatment-related effects on macroscopic pathology in the P or F1 Cohort 1A and 1B animals. The observed findings were considered incidental, commonly observed in this strain of rat, and/or were of similar incidence in control and treated animals. There were no treatment-related macroscopic findings in animals that had reproductive failure or in females with total litter loss.

2) Microscopic examination: Selected microscopic pathology findings from the P and F1 Cohort 1A animals are presented in Table 8. Treatment-related follicular hypertrophy was observed in the thyroid glands and was associated with increased absolute and/or relative thyroid weights in P and F1 animals. The greater incidence of liver hypertrophy in the 1650 ppm F1 males was associated with the observed increases ($p<0.05$) in mean relative (to body) liver weight in this group. Other microscopic findings were noted but occurred in single animals and/or were of similar incidence in control and treated animals, and were not considered related to treatment.

Additionally, the microscopic examination of the testes and the quantitative evaluation of the ovarian follicles and corpora lutea did not show any difference between the 1650 ppm and control groups in the F1 Cohort 1A animals.

TABLE 8. Selected microscopic findings in P and F1 animals. [a]

Tissue / Observation	Nominal dietary concentration (ppm)			
	0	470	825	1650
P Males				
Thyroid gland: [Number examined]	25	25	25	25
Follicular hypertrophy: Total	0	1	5	5
Minimal	0	1	5	5
P Females				
Thyroid gland: [Number examined]	24	24	24	25
Follicular hypertrophy: Total	0	1	2	5
Minimal	0	1	2	3
Mild	0	0	0	2
F1 Cohort 1A Males				
Thyroid gland: [Number examined]	20	20	20	20
Follicular hypertrophy: Total	0	0	0	3
Minimal	0	0	0	3
Spleen: [Number examined]	20	20	20	20
Extramedullary hematopoiesis:Minimal	1	1	3	2
Liver: [Number examined]	20	20	20	20
Centrilobular hypertrophy: Total	0	0	0	6
Minimal	0	0	0	6
F1 Cohort 1A Females				
Thyroid gland: [Number examined]	20	0	0	20
Follicular hypertrophy: Total	1	-	-	2
Minimal	1	-	-	2

a Data were obtained from Appendix 46 on pages 1544, 1547, 1584-1593, and 1621-1629 of the study report.
b 470 and 825 ppm group tissues were not examined (-).

B. OFFSPRING

1. Viability and clinical signs: There were no treatment-related effects on clinical observations in either generation. Incidental clinical signs in the F1 offspring included the following and were noted in four control litters and ten litters from each treatment group: occasional pale/cyanotic/cold pups, hematoma, complete or partial absence of the tail, weakness, thinness, incomplete hair growth, scabs and/or sores. Incidental clinical signs in the F2 offspring (from F1 Cohort 1B parents) included hematoma, weakness, thinness, cold body, incomplete hair growth, teeth marks, abnormal behavior, and/or sores and were noted in 4, 4, 5, and 6 litters, in ascending dose groups. Note to EPA Reviewer: Summary data for the F1 pups were not provided; individual data were included in Appendix 5. The text mistakenly referred to Table 25 and Appendix 25 in the discussion of F1 pup clinical signs, but these included summary and individual data for F2 pups.

Mean litter size and viability (survival) results for the F1 and F2 pups during lactation are summarized in Tables 9a and 9b, respectively. There were no adverse treatment-related effects on parturition parameters in the F1 and F2 litters. Decreased ($p<0.05$) total and mean numbers of implantations in the 1650 ppm F2 group may have been related to treatment but the difference was relatively minor, and there were no differences in related parameters (pups born, liveborn

pups, PND 0 litter size). Post-implantation loss was decreased ($p<0.05$) in the 1650 ppm F1 animals but this is not toxicologically relevant.

TABLE 9a. Litter parameters in the F1 Cohort 1B generation. [a]

Parameter	Nominal dietary concentration (ppm)			
	0	**470 (205-340)**	**825 (365-600)**	**1650 (730-1200)**
Total # litters	25	23	25	25
# litters w/liveborn pups	25	23	25	25
# litters w/stillborn pups	0	1	0	0
# litters w/all stillborn pups	0	0	0	0
Total # implantations	332	294	314	307
Implantations (mean ± SD)	13.3 ± 1.9	12.8 ± 2.7	12.6 ± 2.4	12.3 ± 2.4
Total # pups delivered	300	268	274	294
Mean (± SD) litter size at birth	12.0 ± 2.4	11.7 ± 2.6	11.0 ± 3.1	11.8 ± 2.9
Number stillborn	0	1	0	0
Perinatal loss, PND 1-4	6	4	5	2
PND 5-21	0	0	1	0
Sex ratio, PND 1 (%♂)	46.3	46.9	46.8	48.8
PND 21 (%♂)	46.5	49.0	47.4	48.8
Total # live born, PND 0	300	267	274	294
PND 4 (pre-cull)	294	263	269	292
PND 4 (post-cull)	196	180	188	195
PND 21	196	180	187	195
Mean (± SD) litter size, PND 0	12.0 ± 2.4	11.6 ± 2.6	11.0 ± 3.1	11.8 ± 2.9
PND 1	11.8 ± 2.5	11.6 ± 2.5	10.8 ± 3.2	11.7 ± 2.9
PND 4 (pre-cull)	11.8 ± 2.5	11.4 ± 2.4	10.8 ± 3.2	11.7 ± 2.9
PND 7	7.8 ± 0.8	7.8 ± 0.7	7.8 ± 0.7	7.8 ± 1.0
PND 14	7.8 ± 0.8	7.8 ± 0.7	7.8 ± 0.7	7.8 ± 1.0
PND 21	7.8 ± 0.8	7.8 ± 0.7	7.8 ± 0.7	7.8 ± 1.0
Live birth index (%)	100	99.6	100	100
Number of females with total litter loss	0	0	1	0
Viability index Day 4	98.0	98.5	98.2	99.3
Lactation index Day 21	100	100	99.5	100
Post-implantation loss (mean % ± SD)	10.07 ± 12.15	8.62 ± 7.60	12.38 ± 19.15	5.47 ± 10.88* (↓46)

a Data were obtained from Table 20 on pages 179-181 of the study report. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.

* Significantly different from control; $p<0.05$.

TABLE 9b. Litter parameters in the F2 generation. [a]

Parameter	Nominal dietary concentration (ppm)			
	0	470 (205-340)	825 (365-600)	1650 (730-1200)
Total # litters	19	20	19	19
# litters w/liveborn pups	19	20	19	19
# litters w/stillborn pups	2	2	1	1
# litters w/all stillborn pups	0	0	0	0
Total # implantations	238	240	230	215* (↓10)
Implantations (mean ± SD)	12.5 ± 3.3	12.0 ± 1.9	12.1 ± 2.3	11.3 ± 1.6* (↓10)
Total # pups delivered	221	220	219	204
Mean (± SD) litter size at birth	11.6 ± 2.9	11.0 ± 2.0	11.5 ± 2.2	10.7 ± 1.4
Number stillborn	8	3	2	1
Perinatal loss, PND 1-4	5	2	4	0
PND 5-21	0	0	1	0
Sex ratio, PND 1 (%♂)	50.3	49.5	49.9	48.8
PND 21 (%♂)	48.0	50.9	48.9	49.2
Total # live born, PND 0	213	217	217	203
PND 4 (pre-cull)	208	215	213	203
PND 4 (post-cull)	139	159	152	151
PND 21	139	159	151	151
Mean (± SD) litter size, PND 0	11.2 ± 3.2	10.9 ± 1.9	11.4 ± 2.2	10.7 ± 1.5
PND 1	11.0 ± 3.7	10.8 ± 1.9	11.3 ± 2.2	10.7 ± 1.5
PND 4 (pre-cull)	10.9 ± 3.6	10.8 ± 1.9	11.2 ± 2.1	10.7 ± 1.5
PND 7	7.3 ± 1.8	8.0 ± 0.2	7.9 ± 0.2	7.9 ± 0.2
PND 14	7.3 ± 1.8	8.0 ± 0.2	7.9 ± 0.2	7.9 ± 0.2
PND 21	7.3 ± 1.8	8.0 ± 0.2	7.9 ± 0.2	7.9 ± 0.2
Live birth index (%)	96.4	98.6	99.1	99.5
Number of females with total litter loss	0	0	0	0
Viability index Day 4	97.7	99.1	98.2	100
Lactation index Day 21	100	100	99.3	100
Post-implantation loss (mean % ± SD)	6.52 ± 6.22	8.15 ± 9.45	4.68 ± 5.05	4.81 ± 7.65

a Data were obtained from Table 44 on pages 268-270 of the study report. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.

* Significantly different from control; p<0.05.

2. <u>Pup body weight</u>: Offspring body weight and body weight gain data are presented in Tables 10a and 10b for the F1 and F2 pups, respectively. Treatment-related decreases in pup body weight were evident in pups from the mid- and high-concentration treatment levels- of both generations. Body weights were decreased (p<0.05) by 9-11% and 8-12% (except for NS on PND 4) in the 730-1200 ppm males and females, respectively, in the F1 litters during PND 1-21 and decreased (p<0.05) by 5-14% (PND 4-21) and 5-12% (PND 7-21) in the 730-1200 ppm males and females, respectively, in the F2 litters. Lesser, and less frequent, decreases (p<0.05) of 5-7% were observed in the 365-600 ppm F1 and F2 offspring of both sexes. These decreases were reflected in the concomitant body weight gain values (calculated by the Reviewers) and considered adverse at ≥365-600 ppm in both generations as these decreases generally increased in magnitude during PND 1-21 and achieved levels of >10% in all 730-1200 ppm offspring prior to PND 21. The dose-related decreases in body weight persisted into the postweaning and premating periods of the F1 adults. There were no treatment-related effects on body weight or body weight gain in the 205-340 ppm F1 or F2 offspring.

TABLE 10a. Mean (± SD) pup body weight and body weight gain (g) in the F1 litters. [a]

Observation/Study interval		Nominal dietary concentration (ppm)			
		0	470 (205-340)	825 (365-600)	1650 (730-1200)
Males, n =		**25**	**23-24**	**24-25**	**25**
Body weight	PND 1	6.82 ± 0.89	6.61 ± 0.61	6.40 ± 0.78* (↓6)	6.21 ± 0.83** (↓9)
	PND 4 [b]	10.41 ± 1.76	10.34 ± 1.13	9.85 ± 1.71	9.44 ± 1.41* (↓9)
	PND 7	17.01 ± 2.27	16.59 ± 1.46	15.96 ± 2.01* (↓6)	15.17 ± 1.90** (↓11)
	PND 14	34.24 ± 3.24	32.76 ± 2.25* (↓4)	31.87 ± 2.96** (↓7)	30.37 ± 2.39*** (↓11)
	PND 21	56.18 ± 4.80	54.62 ± 3.16	52.75 ± 4.18** (↓6)	49.87 ± 3.56*** (↓11)
Body weight gain [c]	PND 1-4 [b]	3.59	3.73	3.45	3.23 (↓10)
	PND 4 [d] -7	6.63	6.26 (↓6)	6.09 (↓8)	5.73 (↓14)
	PND 7-14	17.23	16.17 (↓6)	15.91 (↓8)	15.20 (↓12)
	PND 14-21	21.94	21.86	20.88(↓5)	19.50 (↓11)
	PND 1-21	49.36	48.01	46.35 (↓6)	43.66 (↓12)
Females, n =		**25**	**23**	**24-25**	**25**
Body weight	PND 1	6.47 ± 0.86	6.22 ± 0.49	6.16±0.76	5.98 ± 0.85* (↓8)
	PND 4 [b]	9.99 ± 1.75	9.85 ± 1.02	9.59 ± 1.64	9.15 ± 1.35 (↓8)
	PND 7	16.44 ± 2.11	15.99 ± 1.30	15.61 ± 2.02* (↓5)	15.19 ± 3.00*** (↓8)
	PND 14	33.36 ± 3.07	31.86 ± 1.89*(↓5)	31.41 ± 3.10** (↓6)	29.51 ± 1.88*** (↓12)
	PND 21	54.09 ± 4.34	52.57 ± 2.61	51.61± 4.44* (↓5)	48.23 ± 3.03*** (↓11)
Body weight gain [c]	PND 1-4 [b]	3.52	3.63	3.43	3.17 (↓10)
	PND 4 [d] -7	6.45	6.09 (↓6)	5.98 (↓7)	6.00 (↓7)
	PND 7-14	16.92	15.87 (↓6)	15.80 (↓7)	14.32 (↓15)
	PND 14-21	20.73	20.71	20.23	18.72 (↓10)
	PND 1-21	47.62	46.35	45.45	42.25 (↓11)
Combined sexes, n =		**25**	**23-24**	**24-25**	**25**
Body weight	PND 1	6.63 ± 0.84	6.40 ± 0.55	6.27 ± 0.74	6.10 ± 0.83* (↓8)
	PND 4 [b]	10.19 ± 1.72	10.09 ± 1.06	9.66 ± 1.66	9.31 ± 1.37* (↓9)
	PND 7	16.70 ± 2.14	16.28 ± 1.34	15.79 ± 1.97	15.18 ± 2.24** (↓9)
	PND 14	33.76 ± 3.11	32.30 ± 2.02* (↓4)	31.64 ± 3.00** (↓6)	29.94 ± 2.08*** (↓11)
	PND 21	55.04 ± 4.47	53.56 ± 2.76	52.21 ± 4.24** (↓5)	49.06 ± 3.19*** (↓11)
Body weight gain [c]	PND 1-4 [b]	3.56	3.69	3.39	3.21 (↓10)
	PND 4 [d] -7	6.53	6.16 (↓6)	6.08 (↓7)	5.86 (↓10)
	PND 7-14	17.06	16.02 (↓6)	15.85 (↓7)	14.76 (↓13)
	PND 14-21	21.28	21.26	20.57	19.12 (↓10)
	PND 1-21	48.41	47.16	45.94 (↓5)	42.96 (↓11)

a Data were obtained from Table 21 on pages 182-185 of the study report; n = # of litters. Percentage differences from control (calculated by the Reviewers for body weight gain) are presented in parentheses.

b Before standardization (pre-cull).

c Calculated by Reviewer from group means, not subjected to statistical analysis.

d After standardization (post-cull). Post-cull body weight mean values were only slighty different from pre-cull values and are not included in the table.

* Significantly different from control; p<0.05.

** Significantly different from control; p<0.01.

*** Significantly different from control; p<0.001.

TABLE 10b. Mean (± SD) pup body weight and body weight gain (g) in the F2 litters. [a]

Observation/Study interval		Nominal dietary concentration (ppm)			
		0	470 (205-340)	825 (365-600)	1650 (730-1200)
Males, n =		**18**	**20**	**19**	**19**
Body weight	PND 1	6.85 ± 0.66	6.77 ± 0.68	6.51 ± 0.65	6.67 ± 0.48
	PND 4 [b]	10.57 ± 1.39	10.52 ± 1.39	10.01 ± 1.19* (↓5)	10.00 ± 0.66* (↓5)
	PND 7	17.28 ± 02.15	17.21 ± 1.77	16.28 ± 1.78* (↓6)	15.70 ± 0.94*** (↓9)
	PND 14	34.80 ± 2.61	34.48 ± 2.56	32.07 ± 2.94*** (↓8)	29.94 ± 1.73*** (↓14)
	PND 21	56.47 ± 3.93	56.59 ± 4.82	52.38 ± 4.87** (↓7)	49.56 ± 2.57*** (↓12)
Body weight gain [c]	PND 1-4 [b]	3.72	3.75	3.50 (↓6)	3.33 (↓10)
	PND 4 [d] -7	6.63	6.62	6.24 (↓6)	5.67 (↓14)
	PND 7-14	17.52	17.27	15.79 (↓10)	14.24 (↓19)
	PND 14-21	21.67	22.11	20.31 (↓6)	19.62 (↓9)
	PND 1-21	49.62	49.82	45.87 (↓8)	42.89 (↓14)
Females, n =		**19**	**20**	**19**	**19**
Body weight	PND 1	6.35 ± 0.80	6.45 ± 0.77	6.30 ± 0.78	6.48 ± 0.44
	PND 4 [b]	10.01 ± 1.45	10.11 ± 1.47	9.77 ± 1.33	9.87 ± 0.63
	PND 7	16.52 ± 2.08	16.50 ± 1.87	15.90 ± 1.75	15.65 ± 1.00* (↓5)
	PND 14	34.09 ± 2.66	33.14 ± 2.48	31.60 ± 2.86** (↓7)	29.85 ± 1.97***(↓12)
	PND 21	54.62 ± 3.47	53.69 ± 4.45	51.33 ± 4.81** (↓6)	49.45 ± 2.71*** (↓9)
Body weight gain [c]	PND 1-4 [b]	3.66	3.66	3.47 (↓5)	3.39 (↓7)
	PND 4 [d] -7	6.54	6.41	6.09 (↓7)	5.72 (↓13)
	PND 7-14	17.57	16.64	15.70 (↓11)	14.20 (↓19)
	PND 14-21	20.53	20.55	19.73	19.60 (↓5)
	PND 1-21	48.27	47.24	45.03 (↓7)	42.97 (↓11)
Combined sexes, n =		**19**	**20**	**19**	**19**
Body weight	PND 1	6.55 ± 0.75	6.60 ± 0.71	6.40 ± 0.71	6.56 ± 0.44
	PND 4 [b]	10.26 ± 1.38	10.32 ± 1.42	9.90 ± 1.24	9.94 ± 0.63
	PND 7	16.87 ± 2.08	16.87 ± 1.78	16.09 ± 1.72* (↓5)	15.68 ± 0.95** (↓7)
	PND 14	34.43 ± 2.52	33.83 ± 2.45	31.85 ± 2.85*** (↓7)	29.89 ± 1.80*** (↓13)
	PND 21	55.60 ± 3.55	55.15 ± 4.56	51.84 ± 4.74** (↓7)	49.48 ± 2.59*** (↓11)
Body weight gain [c]	PND 1-4 [b]	3.71	3.72	3.50 (↓6)	3.38 (↓9)
	PND 4 [d] -7	6.59	6.52	6.16 (↓7)	5.70 (↓14)
	PND 7-14	17.56	16.96	15.76 (↓10)	14.21 (↓19)
	PND 14-21	21.17	21.32	19.99 (↓6)	19.59 (↓7)
	PND 1-21	49.05	48.55	45.44 (↓7)	42.92 (↓12)

a Data were obtained from Table 46 on pages 274-277 of the study report; n = # of litters. Percentage differences from control (calculated by the Reviewers for body weight gain) are presented in parentheses.
b Before standardization (pre-II).
c Calculated by Reviewer from group means, not subjected to statistical analysis.
d After standardization (post-cull). Post-cull body weight mean values were only slightly different from pre-cull values and are not included in the table.
* Significantly different from control; p<0.05.
** Significantly different from control; p<0.01.
*** Significantly different from control; p<0.001.

3. <u>Developmental landmarks</u>: Mean AG indices in the F1 and F2 offspring on PND 1 are presented in Table 11. In the F1 offspring, there were no evident treatment-related effects; the observed differences from control were unrelated to treatment level. In the F2 offspring, the AG index was decreased (p<0.05) by 4% in the 205-340 ppm males and decreased (p<0.001) by 16-21% and 22-26% in the ≥365-600 ppm males and females, respectively.

There were no treatment-related effects on the apparent number and percentage of F1 or F2 male pups having areolae on PND 13; no areolae were detected in any male pups.

TABLE 11. Mean (± SD) anogenital index (mm/kg) in the F1 and F2 litters. [a]

Group / Observation	Nominal dietary concentration (ppm)			
	0	470 (205-340)	825 (365-600)	1650 (730-1200)
F1 Males, n =	**25**	**24**	**25**	**25**
AG index	2.1 ± 0.2	2.1 ± 0.2	2.3 ± 0.2*** (↑10)	2.1 ± 0.1* (0)
F1 Females, n =	**25**	**24**	**24**	**25**
AG index	1.1 ± 0.2	1.1 ± 0.2* (0)	1.2 ± 0.2	1.1 ± 0.2* (0)
F2 Males, n =	18	20	19	19
AG index	2.4 ± 0.3	2.3 ± 0.3* (↓4)	2.0 ± 0.3*** (↓16)	1.9 ± 0.3*** (↓21)
F2 Females, n =	19	20	19	19
AG index	1.2 ± 0.2	1.3 ± 0.2	1.0 ± 0.1*** (↓22)	0.9 ± 0.2*** (↓26)

a Data were obtained from Tables 23 and 47 on pages 189 and 278 of the study report. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.

* Significantly different from control; p<0.05.

*** Significantly different from control; p<0.001.

4. <u>Sexual maturation</u>: The data pertaining to preputial separation and vaginal opening in the F1 offspring (Cohorts 1A/1B/1C) are presented in Table 12. The day of preputial separation was delayed (p<0.001) by approximately 1.4-2.5 days at all treatment levels in a manner unrelated to dose with the shortest delay in the 730-1200 ppm animals. Body weight on the day of acquisition was decreased (p<0.001) by 8% in the 730-1200 ppm males. These differences in males were considered likely related to treatment despite the absence of a linear, monotonic dose response as that the effect may be endocrine-mediated and the delay in the 730-1200 ppm animals was considered adverse as it occurred in conjunction with decreased body weight in these animals. There were no treatment-related effects on attainment of vaginal opening, but the body weight in the 730-1200 ppm animals was decreased (p<0.001) by 6%.

There were no treatment-related differences between the day of vaginal opening and the first estrus in F1 Cohort 1A females.

TABLE 12. Mean (± SD) offspring sexual maturation data from the F1 offspring. [a]

Observation/Study interval	Nominal dietary concentration (ppm)			
	0	470 (205-340)	825 (365-600)	1650 (730-1200)
Males				
Day of preputial separation	50.0 ± 6.9	52.5 ± 5.9*** (↑)	52.4 ± 6.8***(↑)	51.4 ± 4.1***(↑)
Body weight (g)	235.25 ± 33.68	235.84 ± 32.11	231.42 ± 34.70	216.13 ± 25.27*** (↓8)
Females				
Day of vaginal opening	32.6 ± 2.0	32.6 ± 2.0	32.8 ± 1.7	32.9 ± 2.1
Body weight (g)	107.91 ± 11.48	105.43 ± 11.54	114.87 ± 12.40	100.92 ± 9.34*** (↓6)

a Data were obtained from Tables 38 and 39 on pages 249-252 of the study report; n = 60. Percentage differences from control are presented in parentheses.

*** Significantly different from control; p<0.001.

5. Thyroid hormone analysis: Thyroid hormone data from surplus F1 PND 21 offspring are presented in Table 13. There were no treatment-related effects on T4 concentrations of either sex. Mean serum TSH levels were increased (p<0.05) by 65% in the 730-1200 ppm males and by 68-78% in the ≥365-600 ppm females

In F1 PND 4 pups, the mean T4 levels were comparable to controls (data not shown); TSH was not measured in PND 4 pups.

TABLE 13. Mean (± SD) serum thyroid hormone concentrations in surplus F1 PND 21 offspring. [a]

Parameter	Nominal dietary concentration (ppm)			
	0	470 (205-340)	825 (365-600)	1650 (730-1200)
F1 PND 21 Males				
T4 (µg/dL)	4.62 ± 0.88	5.45 ± 0.75	5.04 ± 0.49	5.65 ± 1.08
TSH (µIU/mL)	0.062 ± 0.032	0.064 ± 0.024	0.073 ± 0.055	0.102 ± 0.038* (↑65)
F1 PND 21 Females				
T4 (µg/dL)	5.08 ± 1.11	4.71 ± 0.72	5.44 ± 0.92	5.78 ± 0.63
TSH (µIU/mL)	0.053 ± 0.016	0.068 ± 0.025	0.089 ± 0.046* (↑68)	0.099 ± 0.050* (↑87)

a Data were obtained from Appendix 45 on page 1522 of the study report; n = 10. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.

* Significantly different from control; p<0.05.

** Significantly different from control; p<0.01.

*** Significantly different from control; p<0.001.

6. Offspring postmortem results:

a. Organ weights: Selected organ weight data from the F1 Cohort Surplus and F2 weanlings terminated on PND 21 are presented in Table 14. Terminal body weights were decreased (p<0.05) by 6-10% in the ≥365-600 ppm F1 and 1650 ppm F2 +males. Absolute and relative (to body) spleen weights were decreased (p<0.05) by 16-29% and 12-21%, respectively, in the ≥205-340 ppm F1 males, by 18-29% and 13-20%, respectively, in the ≥365-600 ppm F2 males, and by 19-22% and 13-15%, respectively, in the ≥365-600 ppm F2 females . Relative (to body) liver weights were increased (p<0.05) by7% in the 1650 ppm F1 males, by 8-9% in the ≥365-600 ppm F1 females, by 8-14% in the ≥365-600 ppm F2 males, and 9-18% in the ≥365-600 ppm F2 females. As these changes were of relatively low magnitude, they were considered adaptive responses and non-adverse. Absolute thymus weights were decreased (p<0.05) by 15-20% in the 730-1200 ppm F1 and F2 high-dose; these differences were considered related to the decreased terminal body weights in these animals as there were no macroscopic correlates.

TABLE 14. Selected mean (± SD) organ weight data from F1 and F2 weanlings. [a]

Parameter	Nominal dietary concentration (ppm)			
	0	470 (205-340)	825 (365-600)	1650 (730-1200)
F1 PND 21 Males				
Terminal BW (g)	55.51 ± 3.96	52.76 ± 4.19	52.21 ± 4.65* (↓6)	49.81 ± 2.66** (↓10)
Rel. liver (%)	4.02 ± 0.29	4.11 ± 0.28	4.08 ± 0.41	4.31 ± 0.20* (↑7)
Abs. spleen (g)	0.310 ± 0.042	0.261 ± 0.051* (↓16)	0.256 ± 0.043** (↓17)	0.221 ± 0.045** (↓29)
Rel. spleen (%)	0.558 ± 0.071	0.491 ± 0.064* (↓12)	0.490 ± 0.059* (↓12)	0.443 ± 0.081** (↓21)
Abs. thymus (g)	0.266 ± 0.033	0.257 ± 0.033	0.247 ± 0.056	0.212 ± 0.028** (↓20)
F1 PND 21 Females				
Terminal BW (g)	50.63 ± 4.94	51.64 ± 4.24	48.83 ± 3.21	48.30 ± 3.90
Rel. liver (%)	3.84 ± 0.20	3.96 ± 0.20	4.14 ± 0.28** (↑8)	4.20 ± 0.24** (↑9)
F2 PND 21 Males				
Terminal BW (g)	59.27 ± 6.51	59.22 ± 7.22	55.83 ± 6.20	53.33 ± 3.85* (↓10)
Rel. liver (%)	4.83 ± 0.46	5.13 ± 0.48	5.23 ± 0.55* (↑8)	5.49 ± 0.45** (↑14)
Abs. spleen (g)	0.318 ± 0.055	0.293 ± 0.057	0.260 ± 0.055* (↓18)	0.227 ± 0.042** (↓29)
Rel. spleen (%)	0.538 ± 0.089	0.495 ± 0.078	0.469 ± 0.099* (↓13)	0.428 ± 0.082** (↓20)
Abs. thymus (g)	0.257 ± 0.038	0.272 ± 0.039	0.264 ± 0.035	0.218 ± 0.028* (↓15)
F2 PND 21 Females				
Terminal BW (g)	57.54 ± 6.09	57.41 ± 5.12	53.81 ± 6.19	52.97 ± 4.27
Rel. liver (%)	4.84 ± 0.51	5.12 ± 0.41	5.29 ± 0.43* (↑9)	5.72 ± 0.82** (↑18)
Abs. spleen (g)	0.309 ± 0.066	0.277 ± 0.063	0.250 ± 0.042** (↓19)	0.241 ± 0.028** (↓22)
Rel. spleen (%)	0.537 ± 0.098	0.480 ± 0.088	0.466 ± 0.062* (↓13)	0.455 ± 0.042* (↓15)

a Data were obtained from Appendix 46 on pages 1645-1648 and 1651-1654 of the study report;n = 10. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.

* Significantly different from control; p<0.05.

** Significantly different from control; p<0.01.

b. Pathology:

1) Macroscopic examination: There were no treatment-related macroscopic findings in the F1 or F2 offspring on PND 21.

2) Microscopic examination: Microscopic examinations were not conducted for F1 and F2 offspring terminated during the postnatal/weaning period.

III. DISCUSSION AND CONCLUSIONS:

A. INVESTIGATORS' CONCLUSIONS: Under the experimental conditions of the study, HCCB administered continuously in the diet to male and female Wistar Han rats at dose levels of 470, 825 and 1650 ppm induced no parental or reproductive toxicity for the P males exposed for 10 weeks prior to mating, during mating, and up to termination or for the P females exposed for 10 weeks prior to mating, gestation, and lactation through weaning. There was no sign of reproductive toxicity for Fl males and Fl females exposed at the same dose levels during approximately 19/20 weeks for Cohort 1B.

A parental and reproduction No Observed Adverse Effect Level (NOAEL) of at least 1650 ppm was derived, corresponding to a minimum achieved intake of 94.1 and 91.7 mg/kg/day in males and females, respectively (considering the lowest HHCB intake prior to mating or during gestation and lactation).

Test item-related preweaning developmental effects consisted in a dose-related lower mean pup body weight in all groups from PND1 for the F1 generation or at 825 and 1650 ppm on PND 21 for both sexes for the F2 generation. This finding was considered not adverse as all mean values on PND21 for both generations remained within or very close to the HCD range. Postweaning developmental effects included a dose-related effect in all groups on mean body weight gain prior to mating (males and females) and during the gestation period (Cohort 1B) associated with a slight reduction in mean food consumption considered not toxicologically significant in view of the low magnitude of the changes and given that terminal mean body weight for males was comparable with the HCD. In addition, the minor differences in mean pup body weight or postweaning mean body weight gains were not associated with any other test-item related developmental effect. A developmental NOAEL of at least 1650 ppm was derived, corresponding to a minimum achieved intake level of 123.2 and 95.2 mg/kg/day in males and females, respectively, considering the lowest HHCB intake during development of the pups).

B. **REVIEWER COMMENTS:** The Reviewers disagree with some of the Investigators' conclusions (and the derived NOAELs and LOAELs)the decreases in absolute body weight and body weight gain were considered adverse in F1 mid- and high-dose males from the postnatal period throughout adulthood, in F2 mid- and high-dose male pups, in F1 high-dose females during gestation, and in F1 and F2 high-dose female pups. The Investigator concluded that decreases in the absolute body weights of the F1 Cohort 1B males were not toxicologically significant because the mean body weight of the 1650 ppm animals on Day 120 (when the largest difference was seen) was comparable to the HCD (432.6 ± 37.76 g).; that the differences in pup body weights at ≥730--1200 ppm were not adverse because they remained within or very close to the HCD range (PND 21: males, 49.7-57.6 g; females, 48.8-56.0 g); that the delays in the achievement of preputial separation in males were incidental because the times of achievement were just slightly greater than the HCD range (41.7-47.5 days), there was no concentration-response relationship, and the differences were of "low magnitude".; and that the differences in AG index values were incidental because they were comparable to the HCD mean values (2.0, males; 1.0, females) and the respective control groups had greater mean values than the HCD.

1. **PARENTAL SYSTEMIC TOXICITY:** There were no treatment-related effects on mortality; clinical signs of toxicity; hematology, clinical chemistry, and urinalysis parameters; splenic lymphocyte subpopulations; and macroscopic pathology. Treatment-related decreases ($p<0.05$) in body weight, body weight gain, and food consumption were observed in the ≥825 ppm adult F1 males throughout the study and in the 1650 ppm adult F1 females during gestation. Observed thyroid-related effects included increased ($p<0.05$) absolute and/or relative thyroid weights in the 1650 ppm P and F1 males and females, the 825 ppm P females and F1 males, and the 430 ppm P females and F1 Cohort 1A males; increased minimal to mild bilateral diffuse follicular cell hypertrophy in the ≥825 ppm P and the 1650 ppm F1 animals of both sexes; and decreased ($p<0.05$) serum T4 concentrations in the ≥825 ppm P and F1 males and increased ($p<0.05$) TSH in

the ≥825 ppm F1 females. Based on the absence of treatment-related lesions in the thyroids and liver weight increases in the 1650 ppm animals (attributed to an adaptive response), the thyroid-related effects are considered as liver-mediated (*e.g.*, treatment-related induction of hepatic enzymes). Therefore, these changes were considered non-adverse.

The parental LOAEL for dietary HHCB in Wistar rats was 825 ppm (equivalent to 45.9/50.6 mg/kg/day in the P males/females during premating) based on decreases in body weight. body weight gain, and food consumption in the F1 males. The NOAEL was 470 ppm (equivalent to 25.8/29.0 mg/kg/day in the P males/females during premating).

2. **OFFSPRING:** There were no adverse, treatment-related effects on viability; clinical signs of toxicity; TSH concentrations; and macroscopic pathology. Treatment-related decreases in pup body weight were evident in offspring at ≥365-600 ppm of both generations. These decreases were reflected in the concomitant body weight gain values and considered adverse. Preputial separation was delayed ($p<0.001$) at all treatment-levels in a manner unrelated to treatment level but the delays were approximately 1.5-2.5 days greater than the concurrent control and approximately 4-11 days greater than the HCD range. The delay in the 730-1200 ppm males occurred concurrently with decreased ($p<0.001$) body weight. Serum TSH concentrations were increased ($p<0.05$) in the 730-1200 ppm F1 males and the 365-600 ppm F1 females on PND 21 (observed effects in the F1 PND 21 females were consistent with the observed effects in the F1 Cohort 1A adult females. As with adults, these differences are likely related to treatment but not adverse.

The offspring LOAEL for dietary HHCB in Wistar rats was 825 ppm (equivalent to 45.6/57.5 mg/kg/day in the P females during gestation/lactation) based on decreased body weights and body weight gains in F1 and F2 offspring. The NOAEL was 470 ppm (equivalent to 26.8/34.4 mg/kg/day in the P females during gestation/lactation).

3. **REPRODUCTIVE TOXICITY:** There were no adverse treatment-related effects on estrous cycle parameters in the P and F1 Cohort 1A/1B females; sperm parameters in the P or Cohort 1A males; mating/fertility indices, pre-coital interval, gestation index in the P or F1 Cohort 1B animals; or differential ovarian follicle count evaluations in the F1 Cohort 1A females. In the F2 offspring, the AG index was decreased ($p<0.05$) by 4% in the 205-340 ppm males and decreased ($p<0.001$) by 16-21% and 22-26% in the ≥365-600 ppm males and females, respectively. Data pertaining to AG indices represent a reproductive effect, and as biomarkers of endocrine-mediated effects, were considered adverse.

The reproductive LOAEL for dietary HHCB in Wistar rats was 825 ppm (equivalent to 45.6/57.5 mg/kg/day in the P females during gestation/lactation) based on decreased anogenital indices in F2 males and females. The NOAEL was 470 ppm (equivalent to 26.8/34.4 mg/kg/day in the P females during gestation/lactation).

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirement (OCSPP 870.3800; OECD 443) for an extended one-generation reproduction study in the rat. Note to EPA Reviewer: The Reviewer notes that the basis for omitting the developmental neurotoxicity and

immunotoxicity cohorts was not provided, and some information required for study reports was redacted. The Reviewer recommends checking an unredacted copy of the report to verify that the use of the historical control data was appropriate.

C. STUDY DEFICIENCIES: The following study deficiencies were noted, but do not alter the conclusions for this study.

- The randomization procedures for allocating adult animals to groups, selecting pups for culling, and assigning offspring to cohorts were not detailed.
- Stability results after storage, as presented, included the deviation from nominal instead of the change from the initial concentration. As the decision to increase the test material concentrations in the treated diets was based on erroneous values, the increases should have been more than sufficient to compensate for anticipated test material loss/degradation.
- Summary data for F1 pups were not provided. The text mistakenly referred to Table 25 and Appendix 25 for both F1 and F2 pups but these included summary and individual data from F2 pups.
- Samples of skeletal muscle were not collected.

EPA Reviewer: Lillie Barnett
RAB1, ECRAD, OPPT (7403M)
EPA Secondary Reviewer: Minerva Mercado Feliciano
RAB4, HED, OPP (7509C)

LILLIE MARIE BARNETT Digitally signed by LILLIE MARIE BARNETT Date: 2026.03.17 17:28:38 -04'00'

MINERVA MERCADO FELICIANO Digitally signed by MINERVA MERCADO FELICIANO Date: 2026.03.19 08:39:18 -04'00'

DATA EVALUATION RECORD

STUDY TYPE: Uterotrophic Assay (Mouse); OCSPP 890.1600; OECD 440

CAS NO: 1222-05-5

TEST MATERIAL (PURITY): HHCB (purity not specified)

SYNONYMS: 1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexamethylcyclopenta- γ-2-benzopyran

CITATION: Seinen, W.S., Lemmen, J.G., Pieters, R.H. H., et al. (1999). AHTN and HHCB show weak estrogenic—but no uterotrophic activity. *Toxicology Letters* 111(1-2), 161-168.

SPONSOR: Not available

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: In a uterotrophic assay conducted to screen for potential estrogenic activity, HHCB (unknown lot number and % purity) was administered daily via animal feed to 6 immature (PND 21), intact female Balb/c mice per dose group at dose levels of 0 (vehicle), 50, or 300 ppm (equivalent to 0, 6 or 40 mg/kg-day) for two weeks. A positive control group of 6 female mice received subcutaneous injections of 0.14 mg 17β-estradiol dissolved in 50 µL corn oil on days 1, 5, 9 and 12 of the experiment. At the end of the 2-week period, animals were necropsied to determine uterine, thymus, and liver weights.

The positive control group showed a 60% increase in mean uterus weight relative to body weight (g/100 g body weight) compared to controls (0.90 ± 0.18 vs. 0.56 ± 0.23; $p < 0.05$). HHCB treatment had no significant effects on uterine weight compared to the vehicle control up to the highest dose tested (0.71 ± 0.32). However, the study has several deficiencies, and two of them are major deficiencies that prevent a clear interpretation of the results:

- Purity information for the test article is missing, making it impossible to know if the lack of activity is due to low purity.

- The study did not adhere to the age range (PND 18-21 and in any case prior to PND 25) and treatment period (3 days) recommended by OECD 440 and OCSPP 890.1600. This means that the experiment, as conducted, lacks the proper sensitivity to detect potential estrogenicity induced by the test compound. Mice can have their first estrus starting at about PND 24, and the fluctuations in natural hormones associated with estrus may confound the perceived effect of the test article and positive control.

This study is classified as **non-guideline but acceptable** for inclusion in weight of evidence discussions. While this study lacks the proper sensitivity to assess the estrogenic potential of the test substance, it is potentially informative for weight of evidence discussion alongside other studies.

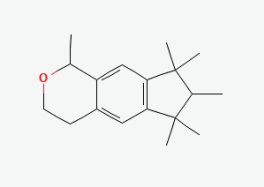
COMPLIANCE: Signed and dated GLP Compliance and Quality Assurance statements were not provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Substance: HHCB

Description: Not provided
Source: International Flavours and Fragrances (IFF), Hilversum, The Netherlands
Lot/Batch #: Not provided
Purity: Not provided
Stability: Not provided
CAS #: 1222-05-5
Structure:



2. Reference Estrogen: 17β-estradiol

Supplier: Sigma, St Louis, MO
Lot/Batch #: Not provided
Purity: Not provided
CAS: 50-28-2

3. Solvent/Vehicle Control (test substance): Acetone

Supplier: Not provided
Lot/Batch #: Not provided
Rationale (if other than water): Not provided [try not to speculate in the public document]
Final concentration: 10 mL added to animal feed and allowed to evaporate although additional details of this procedure were not indicated.

Solvent/Vehicle Control (reference estrogen): Corn oil

Supplier: Not provided
Lot/Batch #: Not provided
Rationale (if other than water): Not provided
Final concentration: 100% v/v, final volume was 50 µL for subcutaneous injection.

4. Test Animals:

Species:	Mouse; female juvenile non-ovariectomized
Strain:	Balb/c
Age/weight at dose initiation:	Mice were 21 days old when obtained but it is unclear whether or how long they were allowed to acclimate prior to dose initiation. Because they were obtained from the same animal facilities at which they were housed, it is likely that dosing started at 21 days. Mean body weight was 10.9–11.4 g.
Source:	Utrecht University
Housing:	Animals were housed at Utrecht University. Additional details (animals/ cage, type of cage, bedding, etc.) were not provided
Diet:	Standard lab chow (Hope Farms B.V., Woerden, The Netherlands) was provided *ad libitum*
Water:	Acidified tap water was provided *ad libitum*
Environmental conditions:	Temperature: 22°C Humidity: 50–60% Air changes: Not provided Photoperiod: 12-h light/dark cycle.
Acclimation period:	Not specified

B. STUDY DESIGN

1. **In Life Dates:** Start: Not specified. End: Not specified.

2. **Study Design:** Immature (probably 21 days old), intact female mice were administered the test substance for 2 weeks. The ages of the animals at the start and end of treatment were not specified; however, because animals were obtained in-house, it is likely that there was no acclimation period and that mice were treated from PND 21 through PND 35. Mice were euthanized at the end of the 2-week treatment period (the precise day after last treatment was not specified.) Uterus, thymus, liver, and body weights were recorded. It is unclear whether the pups were housed with their dam prior to weaning.

3. **Animal Assignment:** Randomized groups of six mice were assigned to the test groups noted in Table 1. Further details on the randomization procedure and group assignment were not provided. It is unclear if animals were within acceptable criteria for weight variability at study initiation. The study did not state whether there were significant differences among group means or whether the body weight of each animal was within 20% of the overall mean.

Table 1. Study Design[a]

Test Group	Dose (ppm); Estimated daily intake (mg/kg-day)	# of Females
Estrogen Agonist Assay		
Vehicle Control	0	12
Low	50 ppm; 6 mg/kg-day	6
High	300 ppm; 40 mg/kg-day	6
17β-estradiol, Reference Estrogen	0.14 mg dissolved in 50 µL corn oil on days 1, 5, 9, and 12	6

[a] Data were obtained from the Methods section of the study.

4. **Dose Selection Rationale:** No rationale or range finding study was described to provide a basis for the selected doses of the test substance.

5. **Dose Preparation and Analysis:** Dose formulations for the test substance were prepared one day before the experiment by initially dissolving the appropriate amounts of test substance in acetone (10 mL total volume) and mixing it thoroughly with feed**.** It is unclear from the study whether the doses were adjusted daily based on individual body weight measurements. Homogeneity and stability were not tested; however, because HHCB is a viscous liquid at room temperature with a high boiling point, it was likely stable in animal feed. The authors did not specify whether samples of dose formulations were analyzed for achieved concentration. It is unclear whether 17β-estradiol was prepared daily in corn oil vs. at one time at the beginning of the study.

   Results of Dose Analysis: Because no information was provided regarding the homogeneity, stability, or achieved concentration, it is uncertain whether the mixing procedure was adequate and whether the variation between nominal and actual dosage to the animals was acceptable.

6. **Dosage Administration:** HHCB was administered to the animals daily via the diet for two weeks. No rationale was provided for this method or duration of administration. 17β-estradiol was given to the positive control group on days 1, 5, 9, and 12 of the experiment via subcutaneous (s.c.) injections of 0.14 mg dissolved in 50 µL corn oil. It is unclear whether injection sites were rotated between six areas on the dorsum to minimize irritation. It is unclear whether dose volumes were adjusted daily based on the most recently recorded body weight.

7. **Statistics:** Means and standard deviations were calculated. The software used for statistical analysis was not provided. Data that were statistically analyzed included terminal body weight and relative (per 100 grams of body weight) uterine weight, relative thymus weight, and liver weight. Differences were calculated using Scheffé's ANOVA and *P*-values less than 0.05 were considered significant. It is unclear from the study whether a test of homogeneity of variances was performed. The test guideline specifies that determination of homogeneity of group variance is needed, and that treatment groups with homogenous variances should be analyzed using analysis of covariance (ANCOVA), using terminal body weight as the co-variable, to determine differences among groups. Since uterus weight was normalized to bodyweight prior to analysis, this is not considered a major deficiency.

C. METHODS

1. **Clinical Examinations:** The study did not provide details regarding whether clinical observations were conducted.

2. **Body Weight:** Animals were weighed at study initiation and twice a week during the two-week treatment period. At the end of the treatment period, terminal body weights were recorded.

3. **Food Consumption:** Food consumption was not measured.

4. **Necropsy and Measurement of Uterine Weight:** At the end of the 2-week treatment period (precise age of animals or time after final administration of the test substance was not specified), all animals were euthanized via decapitation and the relative uterus, thymus, liver, and terminal body weights were recorded. No details were provided regarding the dissection of the uterus, thymus, or

liver, and it is unclear whether wet vs. blotted uterine weights were taken. EPA reviewers could not access a full text version of the methods paper cited by the authors (Thigpen et al., 1987); however, a study by the same group (Thigpen et al., 2002) reported blotted uterine weight-only; thus, it can be presumed that the uterine weights reported in this study were also blotted.

5. Microscopic Examination: Microscopic examinations were not conducted.

II. RESULTS

A. OBSERVATIONS

1. Mortality: All animals survived until scheduled termination.

A. BODY WEIGHT AND WEIGHT GAIN: Only mean terminal body weights were reported and are summarized in Table 2 below. There were no statistically significant changes in mean terminal bodyweight in any of the groups compared to control.

B. UTERINE WEIGHT: Absolute uterine weights were not reported, and it is unclear whether the uterine weights were for wet vs. blotted. EPA reviewers could not access a full text version of the methods paper cited by the authors (Thigpen et al., 1987); however, a study by the same group (Thigpen et al., 2002) reported blotted uterine weight-only; thus, it is possible that the uterine weights reported in this study were also blotted. As shown in Table 2, relative uterine weights in the HHCB treated groups were comparable to the vehicle controls. The test guideline recommends that a negative assay result should be used with caution if control blotted uterine weights are greater than 0.09% of body weight for immature females; however, this could not be confirmed because absolute uterine weights were not reported. Relative uterine weights in the positive control (17β-estradiol)-treated group were increased ($p<0.05$), as expected.

C. THYMUS, and LIVER WEIGHT: Absolute thymus and liver weights were not reported. As shown in Table 2, relative thymus weights in the HHCB treated groups were comparable to the vehicle controls. Relative thymus weights in the 17β-estradiol-treated group were decreased ($p<0.05$). Relative liver weights in the HHCB treated groups increased in the low and high dose groups ($p<0.05$). Relative liver weights in the 17β-estradiol-treated group were comparable to the vehicle controls.

Table 2. Uterine, Thymus, and Liver Weights from Estrogen Agonist Assay in Balb/c Mice[a]

Parameter	Dose (mg/kg/day)											
	Vehicle Control			Low (6)			High (40)			Reference Estrogen [c]		
	N	Mean	SD	N	Mean	SD		Mean	SD	N	Mean	SD
Terminal BW (g)	12	18.0	1.6	6	19.9	0.6	6	19.0	1.6	6	20.5	1.0
Relative Uterine Weight[b] (g/100g b.w.)	12	0.56	0.23	6	0.63	0.21	6	0.71	0.32	6	0.90*	0.18
Relative Thymus Weight (g/100g b.w.)	12	0.51	0.06	6	0.45	0.03	6	0.51	0.09	6	0.27*	0.03
Relative Liver Weight (g/100g b.w.)	12	5.77	0.24	6	6.25*	0.39	6	7.06*	0.23	6	5.70	1.93

[a] Data were obtained from the Results section of the study
[b] It is unclear whether the uterus was wet or blotted
[c] 0.14 mg of 17β-estradiol dissolved in 50 µL corn oil administered via s.c. injection on days 1, 5, 9, and 12
BW= body weight
N= No. of animals in the group
SD = Standard Deviation
* Significantly different from controls at $p<0.05$

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Dietary exposure to HHCB at dose levels of 6 or 40 mg/kg/day had no effect on uterine weights or thymic weights of mice, but did increase liver weights. Therefore, HHCB can be considered to be negative for estrogenicity in the uterotrophic assay. The positive control substance 17β-estradiol elicited the expected increase in uterine weights and decrease in thymic weights. As the mice were not fully immature at the end of the 2-week exposure period, subtle estrogenic effects cannot be fully excluded.

B. AGENCY COMMENTS: HHCB did not increase uterine weights at the doses tested; however, these negative results should be interpreted with caution. The study lacks the appropriate sensitivity to definitively rule out estrogenic effects because it did not adhere to the age range (PND 18-21 and in any case prior to PND 25) and treatment period (3 days) recommended by OECD 440 and OCSPP Guideline 890.1600. Furthermore, phytoestrogen content in food and bedding were not reported and the control uterine weights were not confirmed to be within acceptable ranges, which further reduces the Agency's confidence in the sensitivity of the assay as performed. These, and other limitations, are discussed below. This study cannot serve as a stand-alone assessment of the estrogenic potential of HHCB; however, it is acceptable for use alongside other studies in a weight of evidence discussion regarding the potential for HHCB to act as an estrogen agonist.

C. STUDY DEFICIENCIES:

- Purity information for the test article is missing, making it impossible to know if the lack of activity is due to low purity. This is a major deficiency that prevents interpretation of study results.

- The absolute weights of the uterus were not reported and were not confirmed to be within acceptable ranges of the terminal bodyweight of the animals. The test guideline states that if the control uterine weights are greater 0.09 percent of bodyweight for immature female animals, a negative assay result should be used with caution and various factors such as the age of the animals and dietary phytoestrogens should be scrutinized.
- The age and treatment duration (approximately PND 21-PND 35) falls outside of the recommended timeline that ensures the sensitivity of the assay, both according to the method referenced by the study authors (Thigpen et al. 1987), and according to OECD and EPA test guidelines. Specifically, Thigpen et al. states that "mice should be weaned at 15 days of age and that the bioassay period should be terminated at 7 days, when the mice are 22 days old, for best reproducible results." Similarly, OECD recommends that the treatment should start at PND 21–22 and last for only 3 days (e.g., PND 21–24). This is a major deficiency that prevents interpretation of study results.
- Phytoestrogen levels in food and in bedding were not reported or confirmed to be within acceptable ranges. The test guideline recommends that these levels be reported because phytoestrogens can increase uterine weights in rodents to an extent high enough to interfere with the Uterotrophic Assay.
- No rationale was provided for using dietary administration instead of subcutaneous injection or gavage. The latter methods are preferred by the test guideline to ensure precise dosing. Given that HHCB is primarily used as a fragrance in air care products and cleaning products, inhalation and dermal are more human-relevant relevant exposure routes for this chemical. However, this is not considered a major deficiency, as oral exposure does occur.
- No rationale was provided for using mice instead of rats. According to the standard evaluation protocol, "mice may be used instead of rats in some cases; however, a rationale for this choice should be provided, based on toxicology, pharmacokinetic, or other considerations". Given that similar responses have been shown between the species according to the study protocol, this is not a major deficiency.
- No rationale or range-finding study was provided to support the doses used. The guideline recommends that the highest dose level be at or just below the Maximum Tolerated Dose (MTD).
- The achieved doses of the test substance were not reported or confirmed to be within acceptable ranges of the nominal doses.
- The authors did not specify whether wet or dry-blotted uterine weights were recorded. This is considered a minor deficiency, as both methods are acceptable. However, given the other deficiencies in this study, it is possible that the authors were unfamiliar with the assay guideline and therefore did not understand the importance of consistently choosing one method across all mice.
- The authors did not test the uterine weight data for homogeneity of variance. Additionally, although the test guideline recommends that treatment groups with homogenous variances should be analyzed using analysis of covariance (ANCOVA)using terminal body weight as the co-variable. The authors analyzed uterine weight relative to body weight, and this deficiency is minor.

EPA Reviewer: Lillie Barnett, Ph.D.
RAB1, ECRAD, OPPT (7403M)
EPA Secondary Reviewer: Colleen M. Rossmeisl, DVM
Immediate Office, Antimicrobials Division,
Office of Pesticide Programs (7510M)

Signature: LILLIE MARIE BARNETT Digitally signed by LILLIE MARIE BARNETT Date: 2026.03.11 14:23:30 -04'00'
Date:
Signature: COLLEEN ROSSMEISL Digitally signed by COLLEEN ROSSMEISL Date: 2026.01.15 12:34:32 -05'00'
Date:

DATA EVALUATION RECORD

STUDY TYPE: Skin Sensitization non-guideline (Repeat Insult Patch Test) - Human

CAS NO.: 1222-05-5

TEST MATERIAL (PURITY): Galaxolide (100% neat)

SYNONYMS: 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran (HHCB)

CITATION: IFF. (1973) Galaxolide: Repeated insult patch test. Report number redacted. Unpublished.

SPONSOR: International Flavors & Fragrances (IFF R&D), New York, New York.

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: Galaxolide was examined for the potential to induce irritation and contact sensitization in 42 male and female human subjects (44 subjects entered the study, however 2 withdrew from the study for various reasons). During the induction phase of the study, 0.5 mL of neat galaxolide was applied to a test patch consisting of a 1 x 1-inch Webril swatch affixed to the center of a 1 x 2-inch elastic bandage. The test patches were applied to the upper arms of the subjects in the same location each time unless the severity of a reaction made this inadvisable. Subjects were instructed to remove the patches 24 hours after application, and each site was scored. This was repeated for a total of 9 induction applications. For the challenge application, duplicate patches were applied, one to the original site and one to a skin site which did not previously receive any patches. Both challenge application sites were scored twice, and

duration of the challenge application was not specified.

The schedule of patch application and scoring is redacted in the study report; therefore, it is unclear when the induction and challenge applications occurred and were scored.

Responses to the skin from each patch application were examined and graded using an adapted version of the Draize method, using a 7-point scale provided in the test report. Reactions to the test materials were documented on case report forms by the numerical and letter grade scoring system defined below.

All subjects showed Grade 0 reactions to the treatment during the induction and challenge phases.

Based on the study results, galaxolide is not a dermal irritant or sensitizer when applied neat to human skin.

This study is classified as **acceptable/non-guideline.** The purpose was to provide information on the dermal irritation and sensitization potential of galaxolide in humans, and that purpose was fulfilled. This study is appropriate for qualitative use as part of a weight of evidence determination for the dermal irritation and sensitization potential of galaxolide.

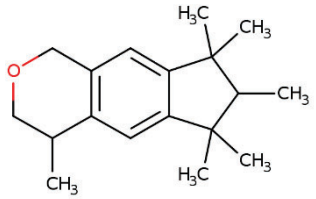
COMPLIANCE: No Data Confidentiality, Quality Assurance, or statements regarding the ethical conduct of the study were provided in the study report.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Materials: Galaxolide
Description: Brown liquid
Lot/Batch #: SC-04-0845
Purity: 100%

CAS # of TGAI: 1222-05-5
Structure:



2. Sample Preparation, Vehicle and/or Positive Control: Test patches were prepared on special order by an undisclosed supplier (redacted) and consisted of a 1 x 1-inch Webril swatch affixed to the center of a 1 x 2-inch elastic bandage. Before application to the skin, 0.5 mL of neat galaxolide was applied to the Webril swatch by means of a calibrated dropper. Vehicle and positive controls were not used in this study. There was no solubility and stability analysis of the test substance.

B. STUDY DESIGN and METHODS:

The objective of the present study was to examine whether galaxolide has the potential to cause allergic contact dermatitis. To this end, human subjects were recruited for repeated insult patch testing with neat galaxolide to determine the presence of irritation and dermal sensitivity.

Study Participants

A total of 43 subjects were originally recruited for this study, however, 2 had to be removed for various reasons (moving, excessive absences). The number of males and females and the ages of participants are summarized in Table 1. A brief medical history was obtained from each subject that included allergies, dermatitides, and concomitant medication. No details were reported regarding criteria for rejecting or withdrawing subjects from the study.

Table 1. Age and Sex Distribution of Subjects.

	16-20 years	21-30 years	31-40 years	41-50 years	51-60years	>60 years	Total
Female	4	10	13	10	1	2	40
Male	0	0	1	0	0	1	2

Institutional Review Board (IRB) Approval and Informed Consent

The study does not report whether written informed consent was obtained from each subject. The study does not mention an IRB approval procedure.

Induction Testing

The following methodology on patch testing is reproduced from the study report (pg. 1):

"The test patches were applied to the upper arms of the panelists. Eight different samples from the group number shown above were tested simultaneously on each panelist. The order of application was varied so that each test material occupied each of the possible positions with approximately equal frequency. The panelists were instructed to remove the patches 24 hours after application. Each patch was applied to the same are each time unless the severity of a reaction made this inadvisable. In such cases, subsequent application was made to an adjacent site or omitted." This was repeated for 9 consecutive applications. Information on when the induction applications occurred relative to one another was redacted from the schedule provided on page 2 of the study report. The duration of time between the final induction application and the first challenge application is also unclear.

Challenge Testing

According to the study report (pg. 1-2), "For the final challenge application duplicate patches were applied, one to the original site and one to a skin site which had not previously received any patches." It is unclear how long samples remained in contact with the skin during the challenge application. Although challenge applications were scored twice, the timing these scorings was not specified.

Rechallenge Testing

No reactions indicative of contact sensitization were observed; therefore, rechallenge testing was not performed on the naïve site for any subjects.

Evaluations

Reactions to the test materials were scored according to the following scale:

0 No evidence of irritation
1 Slight erythema
2 Marked erythema
3 Erythema and papules
4 Edema; erythema may or may not be present
5 Erythema, edema, and papules
6 Vesicular eruption
7 Strong reaction spreading beyond test site
Min Reaction meets min requirements for grade assigned

Effects on superficial layers of the skin were scored as follows:

A Slight glazed appearance
B Marked glazing
C Glazing with peeling and cracking
F Glazing with fissures
G Film of dried serous exudate covering all or portions of the patch site
H Small petechial erosions and/or scabs

No additional details were provided. For example, it is unclear when reactions were scored after each induction application and challenge application because this information was redacted from the schedule provided in the study report (see page 2 of the study report). Additionally, it is unclear whether the same scorer carried out all evaluations of the reaction sites and whether the scorer was blinded as to treatment assignments and any previous scores.

Statistical Analysis

The authors of the study did not perform a statistical analysis of the data.

II. RESULTS

Induction and Challenge Test Results

Figures showing the individual reaction scores and the group totals for the induction and challenge applications can be found on pages 7-9 of the study report. Reaction sites for all subjects received scores of Grade 0 for each induction exposure. Reaction sites for all subjects received scores of Grade 0 for the challenge exposure at both the original and fresh sites.

The authors concluded that 42/42 subjects exhibited “little or no” primary irritation and that 42/42 subjects were “not sensitized”.

III. REVIEWER'S CONCLUSIONS:

The reviewers agree with the study investigators' conclusions. Neat galaxolide does not cause dermal irritation or sensitization in humans, based on the information provided in the study report. The study is appropriate for qualitative use as part of a weight of evidence determination.

IV. STUDY LIMITATIONS/DEFICIENCIES:

- A certificate of analysis confirming the purity and stability of the test substance was not included in the study report.
- Information on the study schedule was redacted, so that the timing of induction and challenge applications and the timing of when reactions were scored were uncertain.
- It is unclear whether the same scorer carried out all evaluations of the reaction sites and whether the scorer was blinded as to treatment assignments and any previous scores.
- Details regarding the recruitment of subjects and ethical conduct of the study were not provided. Some subjects may have been under 18 years of age, it is unclear whether informed consent forms were provided, and no ethical review was mentioned.
- A source for the scale used to grade irritation and sensitization was not provided. The authors state that the procedure was an adaptation of the method of Draize; however, the scale differs from the scoring used in the Draize method.

EPA Reviewer: Lillie Barnett, Ph.D.
RAB1, ECRAD, OPPT (7403M)
EPA Secondary Reviewer: Colleen M. Rossmeisl, DVM
Immediate Office, Antimicrobials Division,
Office of Pesticide Programs (7510M)

Signature: LILLIE MARIE BARNETT Digitally signed by LILLIE MARIE BARNETT Date: 2026.03.11 14:28:13 -04'00'
Date:
Signature: COLLEEN ROSSMEISL Digitally signed by COLLEEN ROSSMEISL Date: 2026.01.15 12:43:32 -05'00'
Date:

DATA EVALUATION RECORD

STUDY TYPE: Skin Sensitization non-guideline (Repeat Insult Patch Test) - Human

CAS NO.: 1222-05-5

TEST MATERIAL (PURITY): Galaxolide (3.75% diluted in 96.25% alcohol SDA 39C)

SYNONYMS: 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran (HHCB)

CITATION: IFF. (1964) Repeated Insult Patch Test of (Redacted). Report number redacted. Unpublished.

SPONSOR: International Flavors & Fragrances, Inc.

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: Galaxolide was examined for the potential to induce irritation and contact sensitization in 47 male and female human subjects, 40 of whom completed the study. During the induction phase of the study, 0.5 mL of galaxolide (3.75% in alcohol SDA 39C) was applied to a test patch consisting of a 1 x 1-inch Webril swatch affixed to the center of a 1 x 3-inch elastic bandage. The test patches were applied to the upper arms of the subjects in the same location each time unless the severity of a reaction made this inadvisable. Subjects were instructed to remove the patches 24 hours after application. This was repeated for a total of 9 induction applications on a Monday-Wednesday-Friday sequence. Sites were scored at the following session. Two weeks after the 9th induction application, a challenge patch was applied to a skin site which did not previously receive any patches. Challenge patches were removed 24

hours after application. Challenge sites were scored 24 hours and 72 hours after patch removal.

Responses to the skin from each patch application were examined and graded using an adapted version of the Draize method, using a 6-point scale provided in the test report. Reactions to the test materials were documented on case report forms.

Out of the 40 test subjects, 7 subjects (17.5%) had reaction sites scored with a Grade of 1 or 2 throughout the induction phase. No reaction sites scored higher than a Grade 2 at any time. For the challenge exposure, reaction sites received Grade 0 for all subjects for both scorings. A separate study examining the vehicle control and/or the use of occlusive patch testing that is attached to end of the report indicates a potential confounding effect of the vehicle control. Specifically, Grade 1-2 reactions occurred in 13-15% of the vehicle-treated population when semi-closed patch testing was used and in 33% when occlusive patch testing was used for the first three induction applications.

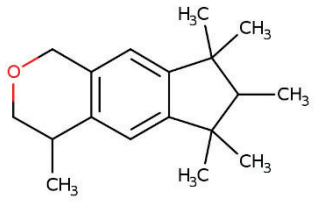
This study is classified as **acceptable/non-guideline.** The purpose was to provide information on the dermal irritation and sensitization potential of galaxolide in humans, and that purpose was fulfilled. This study is considered adequate for qualitative use as part of a weight of evidence determination for the dermal irritation and sensitization potential of galaxolide.

COMPLIANCE: No Data Confidentiality, Quality Assurance, or statements regarding the ethical conduct of the study were provided in the study report.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Materials: Galaxolide
Description: Odorous liquid
Lot/Batch #: Not provided
Purity: 3.75% diluted in alcohol SDA 39C
CAS # of TGAI: 1222-05-5
Structure:



2. Sample Preparation, Vehicle and/or Positive Control: Test patches were prepared on special order by an undisclosed supplier (redacted) and consisted of a 1 x 1-inch Webril swatch affixed to the center of a 1 x 3-inch elastic bandage. Before application to the skin, 0.5 mL of a solution of galaxolide (3.75% dissolved in alcohol SDA 39C) was applied to the Webril swatch. The vehicle was tested in a separate study with a larger sample of participants and the results of this study were attached to the report. The same methodology was used for testing the vehicle control; however, subjects were tested with 2 different bandage protocols: In one protocol, subjects were exposed to an occlusive bandage for 3 days, then a semi-occlusive bandage for the remaining 6 days. In another protocol, subjects were exposed to only a semi-occlusive bandages for all 9 days. A positive control was not used in this study. There was no solubility and stability analysis of the test substance.

B. STUDY DESIGN and METHODS:

The objective of the present study was to examine whether galaxolide has the potential to cause allergic contact dermatitis. To this end, human subjects were recruited for repeated insult patch testing with neat galaxolide to determine the presence of irritation and dermal sensitivity.

Study Participants

A total of 47 subjects were originally recruited for this study; however, 7 had to be removed for various reasons (loss of interest, nausea or respiratory distress attributed to sample odors, unexpected obligations). The number of males and females and the ages of participants are summarized in Table 1. It is unclear whether a medical history was obtained from each subject. No details were reported regarding criteria for rejecting or withdrawing subjects from the study.

Table 1. Age and Sex Distribution of Subjects.

	16-20 years	21-30 years	31-40 years	41-50 years	51-60years	>60 years	Total
Male	3	4	1	1	3	0	12
Female	3	4	10	6	4	1	28

Institutional Review Board (IRB) Approval and Informed Consent

The study does not report whether written informed consent was obtained from each subject. The study does not mention an IRB approval procedure.

Induction Testing

The following methodology on patch testing is reproduced from the study report (pg. 1-2):

"The basic schedule for the large number of tests in the program of which this is a part comprised a series of nine 24-hour exposures on a Monday-Wednesday-Friday sequence for three successive weeks, the reaction to each exposure being scored at the session following and the reaction to the ninth application on Monday of the fourth week. The test patch was applied to the same site each time, unless reaction to sample or tape adhesive rendered this inadvisable, in which case the test patch was either omitted or applied to a fresh site." Information on exactly when the induction and challenge applications occurred for each participant was redacted from the schedule provided on page 2 of the study report.

Subjects 11, 17, 43 and 58 were reported as being tested with closed patches (occluded) for the first 3 applications in induction exposure and "semi-open" (semi-occluded) patches for the remainder of induction and challenge testing. The remaining participants were tested with semi-occluded patches for the entire study.

Challenge Testing

According to the study report (pg. 2), "On Monday of the sixth week a challenge patch was applied to a site not previously exposed and removed after 24 hours. Reactions to challenge were scored on Wednesday and Friday of this week."

Rechallenge Testing

No reactions indicative of contact sensitization were observed; therefore, rechallenge testing was not performed on the naïve site for any subjects.

Evaluations

Reactions to the test materials were scored according to the following scale:

0 No evidence of irritation

1 Slight erythema
2 Marked erythema
3 Erythema and papules
E Erythema and edema
4 Very strong edema and/or papules
5 Vesicular eruption
6 Grade E or stronger reaction spreading beyond test site

No additional details were provided. For example, it is unclear whether the same scorer carried out all evaluations of the reaction sites and whether the scorer was blinded as to treatment assignments and any previous scores.

Statistical Analysis

The authors of the study did not perform a statistical analysis of the data.

II. RESULTS

Induction and Challenge Test Results

Figures showing the individual reaction scores and group totals for the induction and challenge applications involving galaxolide can be found on page 6 of the study report. Out of the 40 test subjects, 7 subjects (17.5%) had reaction sites scored with a Grade of 1 or 2 throughout the induction phase, as shown in Table 1 below. One additional subject was excused from the study due to edematous reactions to the adhesive of the bandage after the fifth application. No reaction sites scored higher than a Grade 2 at any time. For the challenge exposure, reaction sites received Grade 0 for all subjects for both scorings.

Table 1. Total Number of Subjects Exposed to Galaxolide with Grade 1 and 2 Reaction Scores

Subject Number	Grade 1 score	Grade 2 score
11[1]	Days 1, 4, 5, 7 and 9	Day 2
17[1,2]	None	Day 3
58	Day 3	None
92	Days 2 and 4	None
99	Days 7 and 8	None
109	Days 4, 5 and 9	None
151	Day 1	None
166	Days 4, 7 and 9	None

[1] These subjects treated with an occlusive bandage for first 3 induction exposures.
[2] Excused on Day 6 due to reported severe reactions to adhesive material.

Figures showing the individual reaction scores and group totals for the induction and challenge applications from the separate study testing the effects of the vehicle control can be found on pages 9-12 of the study report. In the occlusive followed by semi-open bandage study, 18

subjects out of 55 (33%) reacted when occlusive bandages were used in first 3 days with reaction site scores from Grade 1-3, plus one "E" score. In the last 6 days of that study when the semi-open bandages were used, only 6 subjects out of 47 (13%) had Grade 1-2 reactions. In the vehicle study using only semi-occlusive bandages, 20 subjects (out of 142 initial to 132 final; 14-15%) reacted, with reaction site scores ranging from Grade 1-2. One subject in each of the vehicle control tests received a reaction site Grade 1 score during the challenge exposure; however, neither of these subjects had tested above a Grade 0 during the induction exposure phase.

The authors concluded that "the test sample caused little or no primary irritation" and that "none of the 40 subjects tested was sensitized by the sample." In an additional table summarizing the results of several human sensitization studies that appears at the end of the report, HHCB was described as "minimally irritating" and "non-sensitizing" for this study.

III. REVIEWER'S CONCLUSIONS:

The reviewers generally agree with the study investigators' conclusions. Galaxolide diluted to a final concentration of 3.75 percent in alcohol SDA 39C caused low grade (Grade 1-2) dermal irritation in 7/40 subjects (17.5%) and no sensitization in humans, based on the information provided in the study report. The observed low-grade reactions are likely confounded by potential irritation caused by the vehicle control and/or the use of occlusive patch testing on some subjects. This is supported by vehicle control studies, which showed positive low grade (Grade 1-2) reactions in group sizes ranging from 13-15% of test population when semi-closed patch testing was used exclusively to 33% when occlusive patch testing was used. The study is appropriate for qualitative use as part of a weight of evidence determination.

IV. STUDY LIMITATIONS/DEFICIENCIES:

- A certificate of analysis confirming the purity and stability of the test substance was not provided in the study report.
- It is unclear whether the same scorer evaluated the reaction sites on any group of subjects, and whether scorers were blinded as to treatment assignments and any previous scores.
- A concurrent vehicle control group was not included. However, another report was attached that contains results of a separate study testing the effects of the vehicle control using the same design and methods in 176 participants.
- Details regarding the recruitment of subjects and ethical conduct of the study were not provided. Some subjects may have been under 18 years of age, it is unclear whether informed consent forms were provided, and no ethical review was mentioned.
- A source for the scale used to grade irritation and sensitization was not provided. The authors state that the procedure was an adaptation of the method of Draize; however, the scale differs from the scoring used in the Draize method.
- Information on the closed or occlusive patches vs the semi-open patches was limited to

the vehicle control testing study and did not provide detailed information on either material. Both were referred to as adhesive elastic bandage with the occlusive patch cited as “Elastoplast brand” and the semi-open as “prepared on special order by Duke Laboratories, Inc.” It is assumed the same special order semi-open bandages are used in the test material study, but specific information was redacted.

EPA Reviewer: Lillie Barnett, Ph.D.
RAB1, ECRAD, OPPT (7403M)
EPA Secondary Reviewer: Colleen M. Rossmeisl, DVM
Immediate Office, Antimicrobials Division,
Office of Pesticide Programs (7510M)

Signature: LILLIE MARIE BARNETT Digitally signed by LILLIE MARIE BARNETT Date: 2026.03.11 14:29:58 -04'00'
Date:
Signature: COLLEEN ROSSMEISL Digitally signed by COLLEEN ROSSMEISL Date: 2026.01.15 11:55:47 -05'00'
Date:

DATA EVALUATION RECORD

STUDY TYPE: Skin Sensitization non-guideline (Repeat Insult Patch Test) - Human

CAS NO.: 1222-05-5

TEST MATERIAL (PURITY): Galaxolide (50% diluted in alcohol SDA 39C)

SYNONYMS: 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran (HHCB)

CITATION: IFF. (1973) Repeated Insult Patch Test of (Redacted). Report number redacted. Unpublished.

SPONSOR: International Flavors & Fragrances, Inc. (IFF R&D), New York, New York.

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: Galaxolide was examined for the potential to induce irritation and contact sensitization in 45 male and female human subjects, 43 of whom completed the study. During the induction phase of the study, 0.5 mL of galaxolide (50% in alcohol SDA 39C) was applied to a test patch consisting of a 1 x 1-inch Webril swatch affixed to the center of a 1 x 2-inch elastic bandage. The test patches were applied to the upper arms of the subjects in the same location each time unless the severity of a reaction made this inadvisable. Subjects were instructed to remove the patches 24 hours after application, and each site was scored. This was repeated for a total of 9 induction applications. For the challenge application, duplicate patches were applied, one to the original site and one to a skin site which did not previously receive any patches. Both challenge application sites were scored twice, and duration of the challenge application was not specified.

Responses to the skin from each patch application were examined and graded using an adapted version of the Draize method, using a 7-point scale provided in the test report. Reactions to the test materials were documented on case report forms.

All subjects showed Grade 0 reactions to the treatment during the induction and challenge phases.

Based on the study results, galaxolide is not a dermal irritant or sensitizer when applied neat to human skin.

This study is classified as **acceptable/non-guideline.** The purpose was to provide information on the dermal irritation and sensitization potential of galaxolide in humans, and that purpose was fulfilled. This study is considered adequate for qualitative use as part of a weight of evidence determination for the dermal irritation and sensitization potential of galaxolide.

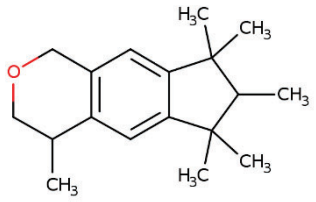
Based on the study results, except for one Grade 1 reaction site reading during induction testing (<1% of panelists), galaxolide caused no primary irritation and no sensitization when applied as a dose of 50% diluted in alcohol SDA 39C to human skin.

COMPLIANCE: No Data Confidentiality, Quality Assurance, or statements regarding the ethical conduct of the study were provided in the study report.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Materials: Galaxolide
Description: Brown liquid
Lot/Batch #: Redacted from report
Purity: 50% in alcohol SDA 39C
CAS # of TGAI: 1222-05-5
Structure:



2. Sample Preparation, Vehicle and/or Positive Control: Test patches were prepared on special order by an undisclosed supplier (redacted) and consisted of a 1 x 1-inch Webril swatch affixed to the center of a 1 x 2-inch elastic bandage. Before application to the skin, 0.5 mL of a solution of galaxolide (50 percent dissolved in alcohol SDA 39C) was applied to the Webril swatch. The vehicle (0.5 mL of 100 percent alcohol SDA 39C) was tested on the same participants using the same procedure and the results of this study were included in a separate attached report. A positive control was not used in this study. There was no solubility and stability analysis of the test substance.

B. STUDY DESIGN and METHODS:

The objective of the present study was to examine whether galaxolide has the potential to cause allergic contact dermatitis. To this end, human subjects were recruited for repeated insult patch testing with neat galaxolide to determine the presence of irritation and dermal sensitivity.

Study Participants

A total of 45 subjects were originally recruited for this study, however, 2 had to be removed for various reasons (excessive absences). The number of males and females and the ages of participants are summarized in Table 1. It is unclear whether all of the subjects were 18 years old or older because ages were reported as ranges. A brief medical history was obtained from each subject that included allergies, dermatitides, and concomitant medication. No details were reported regarding criteria for rejecting or withdrawing subjects from the study.

Table 1. Age and Sex Distribution of Subjects.

	16-20 years	21-30 years	31-40 years	41-50 years	51-60years	>60 years	Total
Female	2	8	11	7	5	3	36
Male	0	2	1	1	2	1	7

Institutional Review Board (IRB) Approval and Informed Consent

The study does not report whether written informed consent was obtained from each subject. The study does not mention an IRB approval procedure.

Induction Testing

The following methodology on patch testing is reproduced from the study report:

"The test patches were applied to the upper arms of the panelists. Eight different samples from the group number shown above were tested simultaneously on each panelist. The order of application was varied so that each test material occupied each of the possible positions with approximately equal frequency. The panelists were instructed to remove the patches 24 hours after application. Each patch was applied to the same are each time unless the severity of a reaction made this inadvisable. In such cases, subsequent application was made to an adjacent site or omitted." This was repeated for 9 consecutive applications. Information on when the induction applications occurred relative to one another was redacted from the schedule provided on page 2 of the study report. The duration of time between the final induction application and the first challenge application is also unclear.

Challenge Testing

"For the final challenge application duplicate patches were applied, one to the original site and one to a skin site which had not previously received any patches." It is unclear how long samples remained in contact with the skin during the challenge application. Although challenge applications were scored twice, the timing of both scorings is also unclear.

Rechallenge Testing

No reactions indicative of contact sensitization were observed; therefore, rechallenge testing was not performed on the naïve site for any subjects.

Evaluations

Reactions to the test materials were scored according to the following scale:

0 No evidence of irritation
1 Slight erythema
2 Marked erythema

3 Erythema and papules
4 Edema; erythema may or may not be present
5 Erythema, edema, and papules
6 Vesicular eruption
7 Strong reaction spreading beyond test site
Min Reaction meets min requirements for grade assigned

Effects on superficial layers of the skin were scored as follows:

A Slight glazed appearance
B Marked glazing
C Glazing with peeling and cracking
F Glazing with fissures
G Film of dried serous exudate covering all or portions of the patch site
H Small petechial erosions and/or scabs

No additional details were provided. For example, it is unclear when reactions were scored after each induction application and challenge application because this information was redacted from the schedule provided in the study report (see page 2 of the study report). Additionally, it is unclear whether the same scorer carried out all evaluations of the reaction sites and whether the scorer was blinded as to treatment assignments and any previous scores.

Statistical Analysis

The authors of the study did not perform a statistical analysis of the data.

II. RESULTS

Induction and Challenge Test Results

Figures showing the individual reaction scores and group totals for the induction and challenge applications can be found on pages 6-8 of the study report. Out of the 43 subjects, only one reaction site received a Grade 1 score (panelist 39 on Day 1); no other Grade 1 or higher scores were recorded for any other reaction sites during the induction test. Reaction sites for all subjects received scores of Grade 0 for the challenge exposure at both the original and fresh sites.

The authors concluded that 43/43 subjects exhibited “little or no” primary irritation and that 43/43 subjects were “not sensitized”.

III. REVIEWER'S CONCLUSIONS:

The reviewers agree with the study investigators' conclusions. Except for one Grade 1 reaction site reading during induction testing (<1% of panelists), galaxolide diluted to a final concentration of 50 percent in alcohol SDA 39C caused no dermal irritation and no sensitization in humans, based on the information provided in the study report. The study is appropriate for qualitative use as part of a weight of evidence determination.

IV. STUDY LIMITATIONS/DEFICIENCIES:

- A certificate of analysis confirming the purity and stability of the test substance were not provided in the study report.
- Details regarding the recruitment of subjects and ethical conduct of the study were not provided. Some subjects may have been under 18 years of age, it is unclear whether informed consent forms were provided, and no ethical review was mentioned.
- It is unclear from the study whether the same scorer evaluated the reaction sites on any group of subjects, and whether scorers were blinded as to treatment assignments and any previous scores. There was no spot checking by an independent/alternative scorer as a quality assurance/quality control measure.
- A source for the scale used to grade irritation and sensitization was not provided. The authors state that the procedure was an adaptation of the method of Draize; however, the scale differs from the scoring used in the Draize method.

EPA Reviewer: Lillie Barnett
RAB1, ECRAD, OPPT (7403M)
EPA Secondary Reviewer: Jessie Wozniak
RAB1, Health Effects Division (7509T)

LILLIE MARIE BARNETT Digitally signed by LILLIE MARIE BARNETT Date: 2026.03.11 17:34:27 -04'00'

JESSIE WOZNIAK Digitally signed by JESSIE WOZNIAK Date: 2026.03.12 08:29:37 -04'00'

DATA EVALUATION RECORD

STUDY TYPE: HHCB Dermal Absorption– Human; OCSPP Non-Guideline

CAS NO.: 1222-05-5

TEST MATERIAL (PURITY): 1,3,4,6,7,8-hexahydro - 4,6,6,7,8,8 - hexamethylcyclopenta - g - 2 - benzopyran (HHCB) (99% radiolabeled)

SYNONYMS: Galaxolide

CITATION: Ford RA, Hawkins DR, Schwarzenbach R, Api AM. The systemic exposure to the polycyclic musks, AHTN and HHCB, under conditions of use as fragrance ingredients: evidence of lack of complete absorption from a skin reservoir. Toxicol Lett. 1999 Dec 20;111(1-2):133-42. doi: 10.1016/s0378-4274(99)00174-5. PMID: 10630708.

SPONSOR: Research Institute for Fragrance Materials, Hackensack, NJ, USA

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/scientific-integrity/epas-scientific-integrity-policy. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: The systemic exposure to HHCB was determined in humans under simulated conditions of exposure. Ring ^{14}C-labeled HHCB was applied without occlusion to three male volunteers at a concentration (4.0 mg/mL in 70% ethanol) approximating that which might be encountered in a typical cologne type product. After a 6-hour exposure period, gauze was removed, and material was washed from the surface of the skin. Skin was tape stripped, covered with fresh gauze, and then tape stripped again at 120 hours. Blood, feces and urine were collected over a 5-day period.

The total absorbed dose was approximately 11.28% for HHCB based on radioactivity from excretion (0.1%), the 6-hour tape strips (10.91%), and the 120-hour tape strips (0.274%). This could under-estimate the total absorbed percentage given that the amount in tissues and deeper layers of the skin is unknown, or it could over-estimate the total absorbed percentage given that the first two tape strips could not be distinguished from the remaining ones as recommended in OECD test guidance 428 for dermal absorption studies.

This study is considered **Acceptable/Non-guideline**. The purpose of this study was to provide an estimate for dermal penetration through human skin. This study is appropriate for qualitative use as part of a weight of evidence determination for the dermal absorption of the test substance.

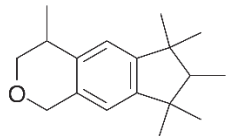
COMPLIANCE: Signed and dated GLP Compliance, Data Confidentiality, and Quality Assurance statements were not included in the published study. The investigators do mention that the study was "conducted with appropriate approval from the Leicester Clinical Research Centre (LCRC) independent ethics committee and the Administration of Radioactive Substances Advisory Committee (ARSAC) of the UK Department of Health and with informed consent of the volunteers."

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Non-radiolabeled TGAI:	HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ] -2-benzopyran and isomers)
Description:	Solid
Batch #:	Not provided
Purity:	98%
CAS # of TGAI:	1222-05-5
Expiration/storage:	Not provided
Structure:	



*Position of radiolabel: Uniformly labeled in the aromatic rings with ^{14}C.

Vehicle/Solvent used:	Ethanol (70% v/v)
Specific Activity:	1.94 mCi/mmol
Radiochemical Purity:	99%
Source:	Wizard Laboratories

2. **Subjects and selection criteria:** The study was conducted in three male human subjects, and no further details including weight, health status, age of the subjects, or selection criteria were provided. The study was carried out at the Leicester Clinical Research Centre (LCRC), Leicester, UK and at Huntingdon Life Sciences, Huntingdon, UK. It was conducted with appropriate approval from the LCRC independent ethics committee and the Administration of Radioactive Substances Advisory Committee (ARSAC) of the UK Department of Health and with informed consent of the volunteers. Subjects remained confined to the clinic from the evening before the dosing until after the 120-h samples were taken. No additional details were provided.

B. STUDY DESIGN AND METHODS

1. **Dose-selection rationale:** HHCB was administered at a concentration that approximates that which might be encountered in a typical cologne type product (4.0 mg/mL). Ethanol was chosen as the solvent since HHCB is found at the highest concentrations in ethanolic products. The investigators noted that initial experiments were conducted in rats to estimate radiological exposure and hence an acceptable amount of radioactivity to administer in the human study.

2. **Dose formulation and administration:** ^{14}C-HHCB was diluted with non-radiolabeled HHCB to a specific activity of 1.94 mCi/mmol. Samples were then dissolved in 70% ethanol to concentrations of 4.0 mg/mL for HHCB. Three male human subjects received a single dermal application of 0.5 mL uniformly spread over a 100 cm2 area of the back. This provided a mean dose of 1.76 mg HHCB (0.018 mg/cm2 body area). After dose application, the solvent was allowed to evaporate (30 min) and the treated area covered with gauze dressing.

3. **Sample collection and preparation:** Total urine passed was collected during the intervals 0–2, 2–4, 4–6, 6–12, 12–24, 24–48, 48–72, 72–96 and 96–120 h after application. Total feces were collected at 24-h intervals for 5 days after application. Blood samples were taken immediately before application and at 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, 96 and 120 h afterwards. A

sample (1 ml) of each blood sample was removed as a whole-blood sample. The remaining sample was centrifuged soon after collection to separate cells from plasma.

Six hours after application, the gauze dressing was removed. Residual HHCB was removed from the skin with cotton wool swabs moistened with 70% aqueous ethanol. Immediately following washing, a 2.5 x 2.5 cm area of treated skin was stripped five times with adhesive tape. The entire treated skin area was recovered with gauze for the rest of the study (6–120 h).

120 hours after dose application, a separate 2.5 x 2.5 cm area of treated skin was stripped five times with successive applications of adhesive tape.

All gauzes, swabs and adhesive tape strippings were retained and stored for analysis. No information was provided regarding the processing, storage, or analysis of urine, fecal, plasma, swab, gauze, and tape strip samples.

4. **Measurement of radioactivity:** Radioactivity was measured by liquid scintillation counting (LSC) using a Model 1409 (Wallac Oy, Turku, Finland) liquid scintillation counter with automatic external standard quench correction. Radioactivity in amounts less than twice background levels was considered to be below the limit of accurate determination.

5. **Evaporation measurement:** A headspace collection chamber (Appendix A) was placed on the forearms of each of two panelists and 20 µL of an 0.5% solution of HHCB in 70% ethanol was uniformly spread over the 5 cm^2 area covered by the chamber. A light airflow, 50 mL/min, was passed through the chamber for 6 h and the headspace collected in a solvent trap (methyl t-butyl ether) and analyzed by GLC for evaporated test material. Notably, these conditions are not comparable to the experimental conditions (0.5 mL of a 0.4% solution of HHCB spread over 100 cm^2).

6. **Calculation of absorption:** The percentage of applied HHCB detected in plasma and excreta were totaled across all measured timepoints to produce the total absorbed percentage.

II. RESULTS

A. **RECOVERY OF RADIOACTIVITY:** Data were expressed as percentage of applied ^{14}C-HHCB and are provided in **Table 1.** Individual and mean totals were summed across all measured timepoints for plasma, urine, and feces. Data from individual timepoints were not provided for these samples. The recovery of ^{14}C-HHCB was below the limit of detection in all samples except for a 12-24 hour urine sample in subject 1. Therefore, the mean percentage in excreta (and thus the mean percentage absorbed past the skin) was calculated to be 0.104%.

The individual and mean totals were combined for the 5 tape strips at 6 hours and again for the 5 tape strips at 120 hours. A 6.25 cm^2 area was tape stripped, and therefore, values were extrapolated to equal the amount of ^{14}C-HHCB in the total 100 cm^2 application area. The mean percentages totaled 10.91% and 0.274%, respectively, for the 6-hour and 120-hour tape strips. Data from individual tape strips were not provided.

The mean percentage of applied ^{14}C-HHCB in the post-dose gauze was 19.54%, and the mean percentage of applied ^{14}C-HHCB in the 6-hour wash plus the final gauze applied after washing was 55.69%. The investigators reported that the mean total recovery was 75.23%. This includes the

percentages of ^{14}C-HHCB in the 6-hour wash plus gauze, in the excreta, and in the post dose gauze. The actual recovery could have been higher because this total does not include the amount lost due to evaporation (estimated to be 22% based on a separate experiment using different conditions), the amounts in upper layers of the skin from tape strips, and any HHCB in deeper layers of the skin or in tissues.

Table 1: Recovery of radioactivity (expressed as percent of applied dose) after 6-hour application of ^{14}C-HHCB to three human volunteers over 24 hours

Recovery of radioactivity after the 6-h application of ^{14}C-HHCB to three human volunteers

Subject	1	2	3	Mean
Dose applied (mg)	1.73	1.79	1.75	1.76
Percent radiolabel recovered in:				
Plasma	ND[a]	ND	ND	
6-h tape strip	0.700 (11.2)[b]	0.649 (10.4)[b]	0.697 (11.1)[b]	0.682 (10.91)
120-h tape strip	0.001 (0.02)[b]	0.011 (0.17)[b]	ND	0.017 (0.274)
Total urine	0.104	ND	ND	
Total feces	ND	ND	ND	
Total excreted	0.104	–	–	
6 h wash plus gauze	48.55	57.11	61.42	55.69
Post dose gauze	22.87	18.67	17.07	19.54
Total recovery	71.42	75.78	78.49	75.23[c]

[a] ND, not detected at limit of detection (generally less than 0.1% depending on sample size).
[b] Calculated amount in stratum corneum from stripping of 6.25 cm^2 of the 100-cm^2 application area.
[c] In a separate experiment under similar conditions it was demonstrated that a mean of 22% of the applied dose evaporated during 6 h.

B. **EVAPORATIVE LOSS:** A mean (n=2 subjects) of 21.8% of the applied dose of HHCB evaporated during the 6-h period. Individual values were not provided. See Appendix A for the headspace collection chamber used to calculate evaporative loss.

III. DISCUSSION AND CONCLUSIONS

A. **INVESTIGATORS' CONCLUSIONS:**
Analyses of excreta of the three volunteers indicated that HHCB was poorly absorbed under the experimental conditions (0.1%). However, tape stripping the upper portion of the stratum corneum demonstrated that a significant amount of HHCB diffused into the skin. Analyses of the radiolabel recovered from the gauze covering the site of application resulted in the recovery of even more (18.9%) of the applied dose at 120 h. The investigators concluded that this was lost from the skin by reverse diffusion, desquamation, or a combination of both.

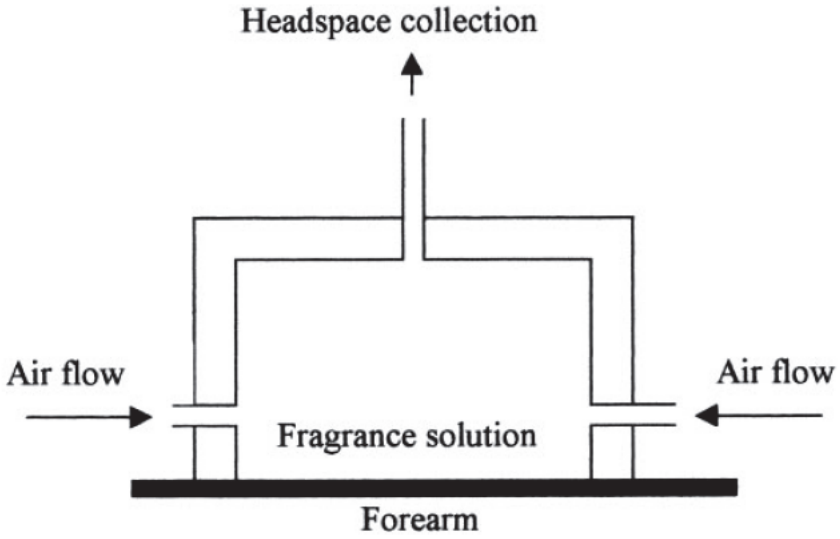
REVIEWER COMMENTS: The Agency agrees with the investigators' general conclusions. However, the amount included in tape strips was not considered as part of the absorbable dose, which is recommended by both the *in vivo* and *in vitro* OECD test guidelines for dermal absorption (OECD 428) in cases where tape strips are combined for analysis. The reviewer concludes that the remaining radioactivity found on the tape strips has the potential to be absorbed over time, and as such, should be considered part of the absorbed dose. The percent recovered radioactivity found on the 6-hour (10.91%) and 120-hour (0.274%) tape strips was added to the total absorbed dose calculated by the investigators (0.1%). Therefore, the total average absorbed dose across human skin was 11.28%. The reviewer

acknowledges that this could over-estimate the total absorbed percentage given that the first two tape strips could not be distinguished from the remaining ones and given that the drop in percentage detected in tape strips at 6 hours versus 120 hours could have been due to reverse absorption into the post-dose gauze rather than absorption into deeper skin layers or tissues. Additionally, the investigators did not acknowledge that the percentage of [^{14}C]-HHCB detected in excreta potentially under-estimates the total absorbed percentage given that the amount in tissues and deeper layers of the skin is unknown. The study is appropriate for qualitative use as part of a weight of evidence determination.

C. STUDY DEFICIENCIES: No major study deficiencies were noted that would alter the conclusions regarding how much HHCB permeated past the skin. However, some methods, data, and uncertainties were not reported. Specifically:

1. The study did not acknowledge the possibility that some of the applied HHCB remained in the body tissues and/or deeper layers of the skin. The percent excreted in this study likely under-estimates the total absorbed dose.
2. The study did not consider [^{14}C]-HHCB measured in tape strips to be included in the total absorbed dose. OECD 428 recommends that the amount in the stratum corneum excluding the first two tape strips (or the total amount in cases where tape strips were pooled), is generally considered absorbable and should be included in the calculation of the dermal absorption value, unless it has been demonstrated that absorption is complete.
3. The study combined tape strips rather than measuring radioactivity for individual tape strips. Therefore, radioactivity from the first two tape strips could not be excluded from the total absorbed dose, which is recommended by OECD 428.
4. Data from individual timepoints were not provided for urine, fecal, and plasma samples.
5. No information was provided regarding the processing, storage, or analysis of urine, fecal, plasma, swab, gauze, and tape strip samples.
6. Limited details were provided regarding the recruitment of the study subjects. Details about age, health, weight, or other demographics were also not provided.
7. The study did not report details regarding the limits of detection for [^{14}C]-HHCB in various media. Specifically, although the investigators reported that radioactivity in amounts less than twice background levels was below the limit of accurate determination, the background levels were not reported.
8. The conditions in the evaporation experiment are not comparable to the experimental conditions (20 µL of an 0.5% solution of HHCB spread over the 5 cm^2 area on the forearm with artificial airflow in n=2 subjects vs. 0.5 mL of a 0.4% solution of HHCB spread over 100 cm^2 on the back covered with gauze in n=3 subjects). Therefore, this is likely not representative of the evaporation that occurred during the permeability experiment.
9. The investigators reported that the mean total recovery was 75.23%. This includes the mean percentages of ^{14}C-HHCB in the 6-hour wash plus gauze, in the excreta, and in the post dose gauze. The study authors did not acknowledge the uncertainty regarding the remaining ~25% of the applied ^{14}C-HHCB, especially given the limitations surrounding the estimated evaporation of the chemical, and the fact that the amount of ^{14}C-HHCB in the skin and tissues is unknown. OECD 427 guideline states that acceptable recovery is 100 ± 10%; recoveries outside of this range must be justified.

Appendix A: Representation of the Headspace Collection Chamber


Headspace collection
Air flow
Air flow
Fragrance solution
Forearm

EPA Reviewer: Lillie Barnett
RAB1, ECRAD, OPPT (7403M)
EPA Secondary Reviewer: Anwar Dunbar
RAB1, Health Effects Division (7509T)

LILLIE MARIE BARNETT Digitally signed by LILLIE MARIE BARNETT Date: 2026.03.11 17:48:48 -04'00'

ANWAR DUNBAR Digitally signed by ANWAR DUNBAR Date: 2026.03.17 08:37:10 -04'00'

DATA EVALUATION RECORD

STUDY TYPE: *In Vitro* Dermal Absorption Study in Humans (OECD 428)

CAS NO.: 1222-05-5

TEST MATERIAL (PURITY): Radiolabeled HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[G]-2-benzopyran and isomers) (99.3% radiochemical purity)

SYNONYMS: Galaxolide

CITATION: An-eX analytical services ltd (2001) In-Vitro Human Skin Penetration Of Radiolabeled Fragrance Material HHCB. Report No: RIFM/4/00, 10091, Redwood Building, Cardiff CF10 3XF, UK.

SPONSOR: Research Institute for Fragrance Materials Inc.

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/scientific-integrity/epas-scientific-integrity-policy. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: In an *in vitro* dermal penetration study, the fragrance [^{14}C]-HHCB (99.3% radiochemical purity, batch/lot 7953-1/010205, specific activity 36.7 mCi/mmol) was applied as a single dermal dose of 20 µL/cm^2 to excised human abdominal and breast skin samples under non-occlusive conditions (198.9 to 203.0 4 µg/cm^2). Twelve horizontal glass diffusion cells and two control cells were prepared with heat-separated epidermal membranes (thickness not reported) and were maintained at 32°C. Membrane integrity was assessed by measuring the permeation rate of tritiated water over a period of one hour. Permeation of HHCB from a 20µL/cm^2 target dose of a 1% solution in ethanol was simultaneously dosed, and then measured over 24 hours, with no post exposure analysis of the skin compartments. The epidermal membranes were then tape stripped 10 times and the radiolabel content of the strips and remaining epidermis were determined. Evaporative loss of HHCB was estimated by measuring the loss from polytetrafluoroethylene (PTFE) sheets under the same experimental conditions.

Following 24 hours exposure, the total absorbed dose was 8.85 % of the applied dose. This total absorbed dose included levels of radioactivity found in tape strips 2-10, any remaining skin after tape stripping, filter paper, and receptor fluid.

This study was conducted prior to publication of the OECD 428 test guideline. The study generally conformed to OECD 428 and is considered **Acceptable/Non-guideline**. The purpose of this study was to provide an estimate for dermal penetration through human skin and this purpose was fulfilled. This study is appropriate for qualitative use as part of a weight of evidence determination for the dermal absorption of the test substance.

COMPLIANCE: A signed and dated Quality Assurance statement was included; however, GLP Compliance and Data Confidentiality statements were not provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[G]-2-benzopyran and isomers)

Description:	Solid
Batch/Lot #:	7953-1/010205
Purity:	98.5%
Compound stability:	Expiry December 2001
CAS # of TGAI:	1222-05-5
Structure:	Not provided in the study report

Vehicle/Solvent used:	Ethanol (96% v/v)
Radiolabeling:	*Position of radiolabel: HHCB-UL-phenyl-^{14}C
Specific Activity:	36.7 mCi/mmol
Radiochemical Purity:	99.3%
Source:	Wizard Laboratories

2. Test skin: Full-thickness human female breast and abdominal skin samples were obtained from cosmetic surgery (supplier unknown). Samples were stored at -20°C and thawed to room temperature for processing. Following removal of the subcutaneous fat by blunt dissection, individual portions of skin were immersed in water at 60°C for 50 seconds. The skin was then pinned, dermis side down, on a cork board and the epidermis (comprising stratum corneum and epidermis) gently removed from the underlying dermis. The dermis was discarded and the epidermal membrane floated onto the surface of water and taken up onto aluminum foil. The membranes were thoroughly dried and stored flat at -20°C until used. The thickness of membranes was not provided.

On the day of use, the epidermal membranes were floated onto water from the aluminum foil and taken up onto 25 mm diameter filter paper supports, ensuring complete coverage of the filter paper. The membranes were then mounted onto diffusion cells. Three different donors were used. The specifications of skin samples are below.

Skin donor number	Sex	Anatomical Region	Age
264	Female	Abdominal	Unknown
265	Female	Breast	28
267	Female	Abdominal	35

B. STUDY DESIGN

1. Dose-selection rationale: No rationale was provided for the target dose, although the authors noted that ethanol was chosen as the solvent since HHCB is found at the highest concentrations in ethanolic products.

2. Doses and Treatment

Nominal doses: 20 µL/cm^2 of 1% (w/v) solution in ethanol

Mean actual dose: 20 µL/cm^2

Volume dispensed: 23 µL (mean)

Duration of exposures (time from dose to skin wash): 24 hours

Termination periods (time from dose to last sampling collection): 24 hours

Number of experimental cells/group: 12 (4 per donor)

3. Experimental setup

Solubility in receptor fluid: The solubility of HHCB in the receptor phase, 6% Volpo N20 in pH 7.4 phosphate buffered saline (Volpo/PBS), was reported to be >2000 µg/mL. Aliquots (1 ml) of radiolabeled HHCB in ethanol solution (1 % w/v HHCB, see below for details of preparation) were transferred to three glass vials (total HHCB per vial was 10 mg). The ethanol was removed under a nitrogen stream, and 4 ml of the receptor phase was added to each vial. The vials were mixed for 24 hours at room temperature. Samples (three per vial) were removed from the solutions and centrifuged (5 min. at 14000 rpm) prior to analysis for radiolabel in triplicate via LSC.

Dose formulation and homogeneity: HHCB was prepared as a 1 % (w/v) solution in ethanol (96% v/v). The study authors stated that ethanol was deemed to be the most appropriate solvent since the highest concentrations of HHCB are found in ethanolic products. The appropriate weight of unlabeled HHCB (246.52 mg) was added to a 25 mL volumetric flask and the radiolabel spike transferred with ethanol washings (calculated radiolabeled HHCB weight 3.31 mg). The volumetric flask was made to volume with ethanol. Total fragrance weight was 249.83 mg and therefore the final solution concentration was 0.999% HHCB (w/v).

The homogeneity was assessed by removing five samples (20 µL) from the prepared radiolabeled vehicle. Aliquots were placed into separate scintillation tubes and counted in a liquid scintillation counter (LSC). The concentration of radioactivity from each of the five regions was within 5% of the mean.

Skin membrane integrity: The integrity of skin samples was checked by measuring the penetration of tritiated water (^3H_2O) through each membrane prior to application of [^{14}C]-HHCB. An aliquot of ^3H_2O (500 µL of 10 µCi/ml) was applied to the surface of the skin membrane. A sample (200 µL) was removed from the receptor phase one hour later. The sample was measured in a LSC. The skin surface was subsequently gently washed seven times and the receptor chambers three times with water. The receptor chamber was then filled with receptor medium (Volpo/PBS) for HHCB permeability testing.

The study authors did not provide a Kp (permeability coefficent) value to be used as a criterion of acceptability (see study deviations). Water permeability was reported for each of the membranes, and no cell exhibited a water permeability greater than 2.1x10^3 cm/h.

Diffusion cell design and set up: Skin samples were mounted as a barrier between the halves of greased horizontal glass Franz-type diffusion cells, with the stratum corneum facing the donor chamber (see Appendix A). The cells were designed such that the area available for diffusion was about 1.0 cm^2 . The skin surface temperature was maintained at 32.0 ± 1 °C by immersing the

diffusion cells in a constant temperature water bath such that the receptor chambers were maintained at 37.0 ± 0.5 °C throughout the experiment. The study authors stated that this method was previously validated (no reference provided). The receptor chamber contents were continuously agitated by small PTFE-coated magnetic followers driven by submersible magnetic stirrers. The receptor chambers were initially filled with a known volume of water, capped, and allowed to equilibrate to the correct temperature.

Administration: [^{14}C]-HHCB was applied to the unoccluded skin membrane with a 25 µL Hamilton syringe at the target dose of ~20 µL/cm^2 exposed skin area. The exact volumes ranged from 19.4 to20.4 µL/cm^2 across the 12 cells (average was 20 µL/cm^2). The HHCB doses ranged from 198.9 to 203.0 4 µg/cm^2 (average was 199.8 4 µg/cm^2).

4. **Sample collection and preparation:** 200 µL samples were taken from each receptor chamber at 1, 2, 6, 12, and 24 hours. Each sample was placed into scintillation fluid and analyzed for ^{14}C by LSC.

   Following the 24-hour receptor phase sample, the diffusion cells were dismantled, and the epidermal membranes were secured onto a small disc of thin plastic using cyanoacrylate adhesive. The fragrance remaining on the skin surface was removed by gentle wiping with a dry cotton bud. The authors did not mention any methods used to confirm full removal of radioactivity, although the recovery was 92% and closer to 100% when adding in the evaporative loss, suggesting that HHCB was removed effectively. Each cotton bud was extracted into 3 mL of methanol and a sample analyzed for radiolabel content.

   Each epidermal membrane was tape stripped 10 times using D-Squame adhesive tape. The tape strips were grouped (placed in the same vial) as follows: Strip 1, strips 2-3, strips 4-6 and strips 7-10. Tape strips were solubilized in 1 mL of OptiSolve® and shaken overnight using a rocker. Samples were subsequently counted. The remaining samples of skin were placed into individual glass vials with 2 mL of OptiSolve® and shaken overnight prior to sampling.

   The diffusion cell donor chambers were wiped to remove sealing grease. The grease wipes were extracted with tetrahydrofuran and a sample counted. The diffusion cell donor chambers were then washed with 15 mL methanol and a sample counted. The filter paper supports were extracted into 2 mL of methanol and a sample counted.

5. **Measurement of radioactivity:** All radioactivity was measured by LSC using a Wallac 1409 scintillation counter. The authors did not state the limit of detection.

6. **Calculation of absorption rate and data processing:** The amount of HHCB per unit area (µg/cm^2) in the receptor phase and various compartments of the skin and diffusion cell were calculated for each cell. The percent of the applied dose of HHCB in those compartments were subsequently calculated and presented. The study authors noted that as per SCCNFP guidelines, the levels of HHCB in the epidermis plus any remaining stratum corneum after tape stripping, filter paper, and receptor fluid were combined to produce a total absorbed dose value.

II. RESULTS

A. **PERMEATION TIME-COURSE DATA:** No radioactivity was detected in the control cells. Individual and mean permeation data at 1, 2, 6, 12, and 24 hours are reported for twelve [^{14}C]-HHCB-dosed cells. Data are expressed as µg/cm^2 and percentage of applied HHCB, respectively, in

Tables 1 and 2. The data show that the radioactivity moved through the skin samples in a time-dependent manner with the maximum amounts occuring at 24 hours for all cells, though they all varied in magnitude.

Table 1: Permeation (expressed as ug/cm^2) of HHCB into the receptor phase from a 20uL/cm^2 target dose of a 1% solution in ethanol over 24 hours.

HHCB dosed cells (HHCB µg/cm^2)

Cell	1 hours	2 hours	6 hours	12 hours	24 hours
1	0.016	0.005	0.025	0.123	0.561
2	0.013	0.023	0.061	0.254	1.043
3	0.004	0.028	0.117	0.454	1.589
4	0.005	0.009	0.058	0.185	0.567
5	0.007	0.008	0.023	0.030	0.196
6	-0.010	0.019	0.037	0.167	0.633
7	0.016	0.037	0.145	0.349	1.035
8	-0.003	0.010	0.018	0.072	0.281
9	-0.005	0.009	0.063	0.243	0.811
10	0.009	0.034	0.061	0.206	0.677
11	0.006	-0.002	0.038	0.205	0.880
12	0.007	0.105	0.302	0.620	1.264
Mean	0.006	0.024	0.079	0.242	0.795
SD	0.008	0.028	0.080	0.165	0.398
SE	0.002	0.008	0.023	0.048	0.115

Data were taken from page 25 of the study report

Table 2: Permeation (expressed as percent of applied dose) of HHCB from a 20µL/cm^2 target dose of a 1% solution in ethanol over 24 hours

HHCB dosed cells (% applied dose permeated)

Cell	1 hours	2 hours	6 hours	12 hours	24 hours
1	0.008	0.002	0.013	0.062	0.281
2	0.006	0.012	0.030	0.128	0.524
3	0.002	0.014	0.058	0.225	0.789
4	0.003	0.005	0.029	0.093	0.284
5	0.003	0.004	0.012	0.015	0.098
6	-0.005	0.010	0.019	0.086	0.327
7	0.008	0.018	0.071	0.171	0.508
8	-0.001	0.005	0.009	0.035	0.140
9	-0.002	0.004	0.032	0.121	0.404
10	0.005	0.017	0.031	0.104	0.341
11	0.003	-0.001	0.019	0.103	0.443
12	0.004	0.052	0.151	0.309	0.630
Mean	0.003	0.012	0.039	0.121	0.397
SD	0.004	0.014	0.040	0.082	0.197
SE	0.001	0.004	0.011	0.024	0.057

Data were taken from page 25 of the study report

B. **DISTRIBUTION DATA FOR ALL COMPARTMENTS AT 24 HOURS:** Individual and mean distribution data at 24 hours are reported for twelve HHCB dosed cells. Data are expressed as

ug/cm^2 and percentage of applied HHCB, respectively, in **Tables 3 and 4.** Following 24 hours exposure, most of the applied HHCB (81.3 ± 2.1%) was found in the 24-hour surface wipe and donor chamber wash plus wipe. The stratum corneum tape strips contained 5.83 ± 0.82% of the applied dose. The epidermis, plus any remaining stratum corneum after tape stripping, contained 4.52 ± 0.57% of the applied dose. The levels of HHCB in the epidermis plus any remaining stratum corneum after tape stripping, filter paper, and receptor fluid were combined by the investigators to produce a total absorbed dose value of 5.16 ± 0.59% of the applied dose. Notably, if the amounts of HHCB detected in tape strips after the first two tape strips are included per OECD TG 428, the absorbed dose value becomes 8.85% of the applied dose. The overall mean recovery of the applied HHCB was 92.1 ± 0.8% and was acceptable (the mass balance). The addition of evaporated HHCB to the above recoveries would move values closer to 100%.

Table 3: Permeation (expressed as µg/cm^2) of HHCB into all compartments from a 20uL/cm^2 target dose of a 1% solution in ethanol at 24 hours

HHCB dosed cells (HHCB µg/cm^2)

Cell No.	Surface wipe	Strip 1	Strips 2-3	Strips 4-6	Strips 7-10	**Remaining skin**	**Receptor phase**	Donor chamber	**Filter paper**	Total recovered
1	108	5.08	4.79	2.12	0.98	**5.24**	**0.561**	58.6	**0.671**	186
2	97	3.35	3.49	2.37	1.90	**8.28**	**1.043**	59.8	**0.821**	178
3	102	5.31	6.84	2.87	1.59	**11.39**	**1.589**	44.0	**0.633**	176
4	97	8.21	10.11	5.08	2.54	**18.91**	**0.567**	31.0	**0.320**	174
5	131	1.04	1.01	0.61	0.39	**3.61**	**0.196**	53.0	**0.146**	191
6	117	3.75	2.18	1.61	1.08	**5.65**	**0.633**	47.7	**0.289**	180
7	126	3.48	3.27	1.70	0.68	**8.50**	**1.035**	45.4	**0.348**	190
8	122	2.68	1.72	0.76	0.47	**7.71**	**0.281**	50.8	**0.230**	187
9	113	4.87	4.29	2.15	1.02	**9.68**	**0.811**	48.0	**0.562**	184
10	135	4.82	3.74	2.70	1.37	**8.07**	**0.677**	32.9	**0.493**	190
11	128	4.72	3.60	1.49	1.18	**11.70**	**0.880**	32.8	**0.669**	185
12	119	4.12	3.96	2.11	0.63	**9.78**	**1.264**	45.6	**0.677**	188
Mean	116	4.29	4.08	2.13	1.15	**9.04**	**0.795**	45.8	**0.490**	184
SD	13	1.73	2.42	1.16	0.63	**3.93**	**0.398**	9.5	**0.216**	6
SE	4	0.50	0.70	0.33	0.18	**1.13**	**0.115**	2.8	**0.062**	2

data in bold typeface are included in the calculation of absorbed dose

Data were taken from page 26 of the study report

Table 4: Permeation (expressed as percent of applied dose) of HHCB into all compartments from a 20µL/cm^2 target dose of a 1% solution in ethanol at 24 hours

HHCB dosed cells (% applied dose)

Cell No.	Surface wipe	Strip 1	Strips 2-3	Strips 4-6	Strips 7-10	Remaining skin	Receptor phase	Donor chamber	Filter paper	Total recovered
1	53.9	2.54	2.40	1.06	0.49	**2.62**	**0.281**	29.3	**0.336**	93.0
2	49.0	1.68	1.75	1.19	0.96	**4.16**	**0.524**	30.1	**0.413**	89.7
3	50.6	2.64	3.40	1.43	0.79	**5.65**	**0.789**	21.8	**0.314**	87.5
4	48.6	4.11	5.06	2.54	1.27	**9.46**	**0.284**	15.5	**0.160**	87.0
5	65.6	0.52	0.51	0.31	0.20	**1.80**	**0.098**	26.5	**0.073**	95.6
6	60.3	1.94	1.13	0.63	0.56	**2.92**	**0.327**	24.6	**0.149**	92.8
7	61.8	1.71	1.61	0.83	0.33	**4.17**	**0.508**	22.3	**0.171**	93.4
8	60.7	1.33	0.85	0.38	0.23	**3.83**	**0.140**	25.2	**0.114**	92.7
9	56.2	2.43	2.14	1.07	0.51	**4.82**	**0.404**	23.9	**0.290**	91.8
10	68.0	2.42	1.88	1.36	0.69	**4.06**	**0.341**	16.6	**0.248**	95.5
11	64.2	2.37	1.81	0.75	0.59	**5.88**	**0.443**	16.5	**0.336**	92.9
12	59.5	2.05	1.97	1.05	0.31	**4.87**	**0.630**	22.7	**0.337**	93.5
Mean	58.2	2.15	2.04	1.07	0.578	**4.52**	**0.397**	22.9	**0.245**	92.1
SD	6.5	0.86	1.21	0.58	0.315	**1.96**	**0.197**	4.8	**0.108**	2.7
SE	1.9	0.25	0.35	0.17	0.091	**0.57**	**0.057**	1.4	**0.031**	0.8

data in bold typeface are included in the calculation of absorbed dose

Data were taken from page 26 of the study report

C. <u>EVAPORATIVE LOSS:</u> The assessment of evaporation of HHCB from PTFE sheets is presented in **Table 5.** HHCB showed 97.6% recovery from the PTFE and donor chamber, which indicated an evaporative loss of 2.4% (**Table 5**).

Table 5: Recovery of HHCB (% applied dose) from PTFE sheet and cell donor chamber

Time after application (h)	% applied HHCB recovered
1	100.9
2	100.4
4	100.2
6	99.3
12	99.1
24	97.6

Data were taken from page 27 of the study report

III. DISCUSSION AND CONCLUSIONS

A. **<u>INVESTIGATORS' CONCLUSIONS</u>:** The results of this study indicate that the level of *in-vitro* percutaneous absorption of HHCB was low, with the majority of the applied HHCB remaining at the skin surface. Overall recoveries of the applied HHCB were 92.1 ± 0.8%. The addition of evaporated HHCB to the above recoveries would move values closer to 100%. The levels of HHCB in the epidermis, plus any remaining stratum corneum after tape stripping filter paper and receptor fluid were combined to produce a total absorbed dose value of 5.16 ± 0.59% of the applied dose.

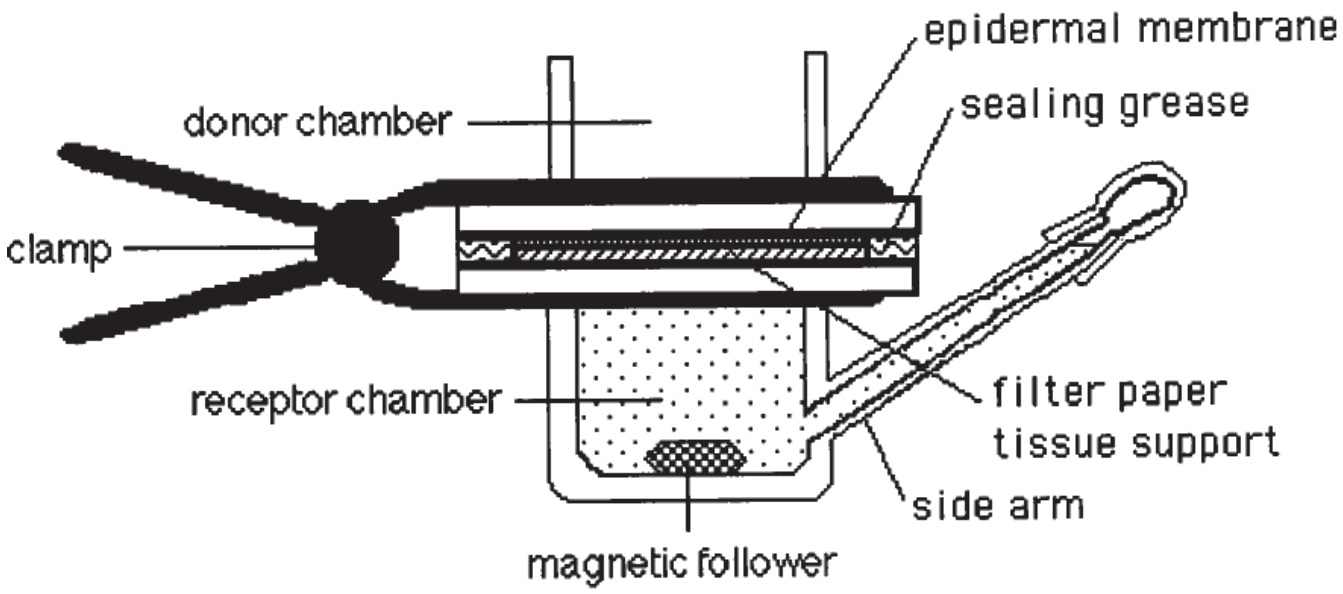
The results of this study were compared to published data from one *in vivo* rat and human subjects skin absorption study conducted in 1999. The overall conclusions were that permeation through human skin was low, although a significant amount of the applied dose was absorbed into the skin. The data from the current study agrees with the published data.

B. **REVIEWER COMMENTS:** The Agency agrees with the investigators' general conclusions. However, tape strips 3-10 were not considered as part of the absorbable dose. The reviewer concludes that the remaining radioactivity found on tape strips 3-10 has the potential to be absorbed over time, and as such, should be considered part of the absorbed dose. Because tape strips 2 and 3 were combined and therefore radioactivity from each could not be distinguished, the remaining radioactivity found on tape strips 2-10 was added to the total absorbed dose calculated by the investigators. Therefore, the total absorbed dose across human skin was 8.85%. This total absorbed dose included radioactivity found in the filter paper, receptor, remaining in skin, and on tape strips 2-10.

C. STUDY DEFICIENCIES: No major study deficiencies were noted that would alter the conclusions. However, this study was conducted prior to the development of OECD TG 428, and therefore, deviated from the testing guidance in the following ways:

1. The study used epidermal membranes prepared from three donors. The test guideline recommends 8 replicates from at least 4 different donors. The age of one of the donors is unknown.
2. The study did not provide a value with which to compare the results of the membrane integrity assessments. However, no cell exhibited a tritiated water permeability greater than $2.1x10^{3}$ cm/h, and this is below thresholds reported in the literature (a 'limit value' for a maximum Kp of 4.5×10^{-3} cm/h and a mean Kp of 2.5×10^{-3} cm/h) for human *ex vivo* skin.
3. The target dose (20 $\mu L/cm^2$) exceeds the dose range recommended by the test guideline (up to 10 mg/cm2 or I0$\mu L/cm^2$). If this dose represents human exposure, a justification was not provided.
4. The study sampled receptor fluid at five timepoints, which is lower than the 6-12 sampling times recommended by the test guideline.
5. HHCB was removed from the skin via a dry cotton bud rather than washed with a cleansing agent. The dry swab with no solvent may result in more chemical remaining on the site. If this method of removal is more consistent with the expected use condition, a justification was not provided.
6. The study did not report a limit of detection for [^{14}C]-HHCB. The study did not consider [^{14}C]-HHCB measured in tape strips 3-10 to be included in the total absorbed dose. OECD TG 428 recommends that the amount in the stratum corneum excluding the first two tape strips, as well as all the material retained in deeper layers, is generally considered absorbable and should be included in the calculation of the dermal absorption value, unless it has been demonstrated that absorption is complete.

Appendix A: Representation of the Horizonal Glass Diffusion Cell


epidermal membrane
donor chamber
sealing grease
clamp
receptor chamber
filter paper
tissue support
side arm
magnetic follower

EPA Reviewer: Lillie Barnett
RAB1, ECRAD, OPPT (7403M)
EPA Secondary Reviewer: Jessie Wozniak
RAB1, Health Effects Division (7509T)

LILLIE MARIE BARNETT Digitally signed by LILLIE MARIE BARNETT Date: 2026.03.11 17:13:10 -04'00'

JESSIE WOZNIAK Digitally signed by JESSIE WOZNIAK Date: 2026.03.12 08:30:32 -04'00'

DATA EVALUATION RECORD

STUDY TYPE: *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method; OECD 439

CAS NO.: 1222-05-5

TEST MATERIAL (PURITY): HHCB/Galaxolide undiluted (70.76%)

SYNONYMS: 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran (HHCB); galaxolide

CITATION: IFF. (2020) HHCB/Galaxolide undiluted: *In Vitro* EPISKIN ™ Skin Irritation Test. Report number redacted. MRID not provided. Unpublished.

SPONSOR: International Flavors & Fragrances I.F.F. (Nederland) B.V., Hilversum, Netherlands

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA's Scientific Integrity Policy for Transparent and Objective Science*, and EPA's Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA's Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA's Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA's Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: The skin irritation potential of HHCB was evaluated using the EPISKIN ™ reconstructed human epidermis model. Tissues (n = 3 per treatment group) were treated with the test item for 15 minutes, rinsed, and incubated for 42 hours. At the end of the post-exposure incubation period, cytotoxicity was measured using the colorimetric MTT reduction assay, which measures cell viability by enzymatic reduction of the yellow MTT tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) to a blue/purple formazan salt within the mitochondria of viable cells. The relative mean viability of the HHCB-treated tissues was 76.9%. HHCB was classified as non-irritant.

This study is classified as **Acceptable/Guideline** and satisfies the requirements for the *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method (OECD 439). This study is appropriate for qualitative use as part of a weight of evidence determination for the dermal irritation potential of the test substance.

COMPLIANCE: Signed and dated GLP Compliance and Quality Assurance statements were provided; however, the names and dates were redacted. All pages of the report included the following information in a footer: "The posting of this document to a public docket maintained by a governmental agency is not a waiver of ownership rights of International Flavors and Fragrances (IFF). Any use of the document by any other person without express written permission would constitute a violation of IFF's rights and subject that person to civil liability. Redactions are claimed as confidential business information (CBI) in accordance with 40 C.F.R. Section 702.37(d)."

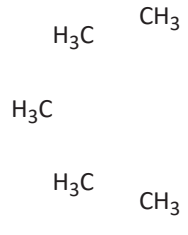
I. MATERIALS AND METHODS

A. MATERIALS:

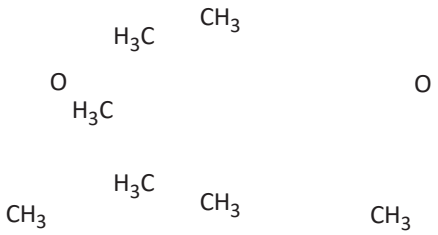
1. Test Materials: HHCB/Galaxolide undiluted
Description: Clear colorless viscous liquid
Lot/Batch #: 0011584169
Purity: 70.76%
Expiration/Storage:

CAS # of TGAI: 1222-05-5; 1222-05-5; 252933-49-6 (Constituent 1); 252933-48-5 (Constituent 2)

Structure:



Constituent 1



Constituent 2

A certificate of analysis and substance identify information document were provided in Annex 1 and Annex 2 of the study report, respectively.

2. Sample Preparation, Negative, and/or Positive Control: HHCB was used as supplied. Dulbecco's Phosphate Buffered Saline (DBPS) with Ca ++ and Mg ++ (batch 2026771; purity 98%) was used as a negative control. DBPS was used as supplied.

Sodium dodecyl sulphate (SDS) (batch 1869697; purity ≥ 99%) was used as a positive control. SDS was prepared as a 5% w/v aqueous solution and was formulated within 2 hours of being applied to the test system.

MTT was prepared as a 3 mg/mL stock solution in DPBS. The stock solution was diluted to 0.3 mg/mL with assay medium when required.

A 0.04 N solution of hydrochloric acid in isopropanol was prepared when required.

3. Test System: EPISKIN ™ Reconstructed Human Epidermis Model Kit
Supplier: EpiSkin Laboratories, Lyon France
Date Received: 21 July 2020
EpiSkin™ Tissues Lot Number: 20-EKIN-030
Maintenance Medium Lot 20-MAIN3-020

Number:
Assay Medium Lot Number: 20-ESSC-020

A technical data, safety sheet and certificate analysis were provided in Annex 4 of the study report. These provided histology (HES stained parrafin section) confirming a multi-layered, highly differentiated epidermis consisting of organized basal, spinous, and granular layers, and a multilayered stratum corneum. Additionally, the barrier function of the specimen was demonstrated (IC_{50} of SDS was 2.5 mg/mL, which falls within the range specified by the manufacturer and within the range specified by the test guideline.

B. STUDY DESIGN AND METHODS:

Test for Direct MTT Reduction

The test item was checked for the ability to directly reduce MTT according to the following procedure. 10 µL of the test item was added to 2 mL of 0.3 mg/mL MTT solution freshly prepared in assay medium. The solution was incubated in the dark at 37°C, 5% CO_2 for 3 hours. Untreated MTT solution was used as a control.

Test for Color Interference

10 µL of test item was added to 90 µL of sterile water. After mixing for 15 minutes on a plate shaker, a visual assessment of the color was made.

Tissue Preparation

On the day of tissue arrival, each tissue was inspected for any air bubbles between the agarose gel and the insert before removal from the transport plate. The investigators reported that the tissues were satisfactory (Annex 4), the temperature indicator color was satisfactory, and the agar medium color was satisfactory.

Maintenance medium was warmed to 37°C and was pipetted into the first column of 3 wells of a pre-labeled 12-well plate. Each epidermis unit was transferred into the maintenance medium-filled wells (3 units per plate). Separate plates were used for the test item, negative, and positive controls. The tissues were incubated at 37°C and 5% CO_2 overnight.

Application of Test Item and Rinsing

The following day, 2 mL of pre-warmed maintenance medium was pipetted into the second column of 3 wells of each 12-well plate.

Tissues (n = 3 per treatment group) were treated with 10 µL (26.3 µL/cm^2) the test item, negative control, or positive control for 15 minutes. This volume ensured uniform covering. In the case of the positive control wells, SDS solution was spread over the entire surface of the epidermis using a pipette tip taking particular care to cover the center. After a 7-minute contact

time, the SDS solution was re-spread with a pipette tip to maintain the distribution of the SDS for the remainder of the 15-minute contact period. Re-spreading was not required for the negative control or test item. Throughout the 15-minute treatment period, plates were kept in the biological safety cabinet at room temperature.

After the 15-minute exposure period, each tissue was removed from the well using forceps and rinsed using a wash bottle containing DPBS with Ca++ and Mg++. Rinsing was achieved by filling and emptying each tissue insert for approximately 40 seconds using a constant soft stream of DPBS to gently remove any residual test item. The rinsed tissues were transferred to the second column of 3 wells containing 2 mL of maintenance medium in each well. The rinsed tissues were incubated at 37° C, 5% CO_2 for 42 hours.

MTT Loading/Formazan Extraction
Following the 42-hour post-exposure incubation period each 12-well plate was placed onto a plate shaker for 15 minutes to homogenize the released mediators in the maintenance medium. 1.6 mL of the maintenance medium from beneath each tissue was transferred to pre-labeled micro tubes and stored in a freezer (-35 to -10°C) for possible inflammatory mediator determination.

2 mL of a 0.3 mg/mL MTT solution freshly prepared in assay medium was pipetted into the third column of 3 wells of the 12-well plates. The tissues were transferred to the MTT filled wells, being careful to remove any excess maintenance medium from the bottom of the tissue insert by blotting on absorbent paper. The tissues were incubated for 3 hours at 37°C and 5% CO_2. At the end of the 3-hour incubation period, each tissue was placed onto absorbent paper to dry. A total biopsy of the epidermis was made using the EPISKIN™ biopsy punch. The epidermis was carefully separated from the collagen matrix using forceps and both parts (epidermis and collagen matrix) placed into labeled 1.5 mL micro tubes containing 500 µL of acidified isopropanol, ensuring that both the epidermis and collagen matrix were fully immersed. Each tube was plugged to prevent evaporation and mixed thoroughly on a vortex mixer. The tubes were refrigerated at 2 to 10° C until Day 6 of the experiment, allowing the extraction of formazan crystals out of the MTT-loaded tissues.

Absorbance/Optical Density Measurements
After formazan extraction, each tube was mixed thoroughly on a vortex mixer to produce a homogenous colored solution. For each tissue, duplicate 200 µL samples were transferred to the appropriate wells of a pre-labeled 96-well plate. 200 µL of acidified isopropanol alone was added to the two wells designated as “blanks”. The optical density (OD_{570}) was measured at 570 nm without a reference filter using the Labtech LT-4500 microplate reader. Servicing, calibration, pass band width and linearity range of the microplate reader were provided in Annex 3 of the study report.

Data Evaluation

The relative mean tissue viabilities obtained after the 15-minute exposure period followed by the 42-hour post-exposure incubation were compared to the mean of the negative control-treated tissues. Relative mean viabilities were calculated in the following way:

$$Relative\ mean\ viability\ (\%) = \frac{Mean\ OD_{570}\ of\ test\ item}{Mean\ OD_{570}\ of\ negative\ control} \times 100$$

Classification of irritation potential was based according to the following table:

Table 1. Classification of Irritation Potential from Relative Mean Viability

Criteria for *in vitro* Interpretation	**Prediction**	**EU CLP (Regulation (EC) No 1272/2008)**	**UN GHS**
Relative mean tissue viability is ≤50%	Corrosive or Irritant	H314 or H315 Category 1 or 2	H314 or H315 Category 1 or 2
Relative mean tissue viability is >50%	Non-irritant	Not classified for irritation	Not classified or UN GHS Category 3 can not be determined

If the relative mean tissue viability is ≤50%, differentiation between EU CLP/UN GHS Category 1 and Category 2 will not be possible based on the results of this study.

Acceptance Criteria

The criteria for an acceptable test required that:

1) The relative mean tissue viability for the positive control treated tissues is ≤ 40% relative to the negative control treated tissues, and the standard deviation value of the percentage viability is ≤ 18%.
2) The mean OD570 for the negative control treated tissues must be ≥ 0.6 and ≤ 1.5, and the SD value of the percentage viability must be ≤ 18%.
3) The SD calculated from individual percentage tissue viabilities of the three test item-treated tissues must be ≤ 18%.

II. RESULTS

Direct MTT Reduction

The investigators reported that the MTT solution containing the test item did not turn blue or purple, indicating that the test item did not directly reduce MTT.

Assessment of Color Interference with the MTT Endpoint

The solution containing the test item was colorless. The investigators concluded that it was therefore unnecessary to run color correction tissues

Test Item, Positive Control Item, and Negative Control Item

The individual and mean OD_{570} values, standard deviations, and tissue viabilities for the test item, negative control, and positive control are provided in Table 2 below. The mean viabilities and standard deviations of the test item and positive control, relative to the negative control are also shown.

The relative mean viability of the test item-treated tissues was 76.9% relative to negative control-treated tissues. Therefore, based on the classification criteria outlined in Table 1, HHCB is classified as a non-irritant. This is also consistent with the criteria outlined in the OECD test guideline.

Table 2. Mean OD_{570} Values and Viabilities for the Negative Control, Positive Control, and Test Item

Item	OD_{570} of tissues	Mean OD_{570} of triplicate tissues	± SD of OD_{570}	Relative individual tissue viability (%)	Relative mean viability (%)	± SD of Relative mean viability (%)
Negative Control Item	0.608	0.712	0.097	85.4	100*	13.7
	0.729			102.4		
	0.800			112.4		
Positive Control Item	0.067	0.049	0.018	9.4	6.8	2.5
	0.048			6.7		
	0.031			4.4		
Test Item	0.603	0.548	0.060	84.7	76.9	8.4
	0.556			78.1		
	0.484			68.0		

OD = Optical Density
SD = Standard Deviation
* = the mean viability of the negative control tissues is set at 100%
Data taken from page 18 of MRID 8785661

Acceptance Criteria

The test acceptance criteria outlined by the investigators, which were consistent with the OECD test guideline, were met. Specifically, the relative mean tissue viability for the positive control-treated tissues was 6.8% relative to the negative control tissues. The mean OD_{570} for the negative control-treated tissues was 0.712. The standard deviations of the relative mean viability for positive and negative control-treated tissues were 2.5 and 13.7%, respectively. There was acceptable variability between tissue replicates for the test chemical, positive control, and negative control (*i.e.,* standard deviations were ≤ 18%).

III. EPA REVIEWER'S CONCLUSIONS:

The reviewers agree with the study investigators' conclusions. Galaxolide (~70% purity) does not cause dermal irritation in human skin, based on the information provided in the study report. The study is appropriate for qualitative use as part of a weight of evidence determination.

IV. STUDY LIMITATIONS/DEFICIENCIES:

No study deficiencies were noted that would alter the conclusions. However, the reviewers do note that the reported purity of HHCB was 70%, which leaves some uncertainty regarding the irritation potential of neat (100%) HHCB.